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## Introduction

Most prostate cancer patients respond initially to androgen ablation and antiandrogen therapy. However, virtually all patients will relapse due to acquisition of the growth of the androgen-independent tumor cells. The molecular mechanism characterizing prostate cancer progression from androgen-dependence to androgen-independence is incompletely understood. We propose that Signal Transducers and Activators of Transcription 3 (Stat3) both regulates the expression of Stat3 target genes, and interacts with AR in prostate cancer cells. The experiments proposed in this application are based upon the hypothesis that Stat3 activation alters androgen receptor signaling pathways, that in turn results in the loss of growth control in prostate cancer cells. We propose to determine the consequence of Stat3 activation in prostate cancer cell growth and to determine the molecular basis of Stat3 interactions with androgen receptor signaling.

## Body

**Since the approval of this application, we have made significant progress of task 1 (i.e., to examine the role of Stat3 activation in prostate cancer cells (months 1-18).**

Task 1A. To establish a series of prostate cancer cell lines demonstrating constitutive Stat3 activation (months 1-6). We have established Stat3 constitutively activated cell sublines in LNCaP cells. These cells express activated Stat3 as examined by EMSA using Stat3 consensus binding sequences (Appendix 1).

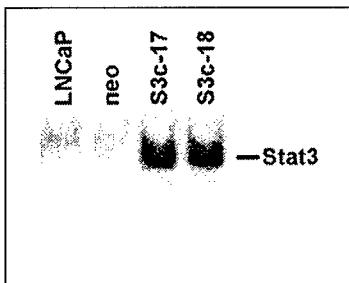
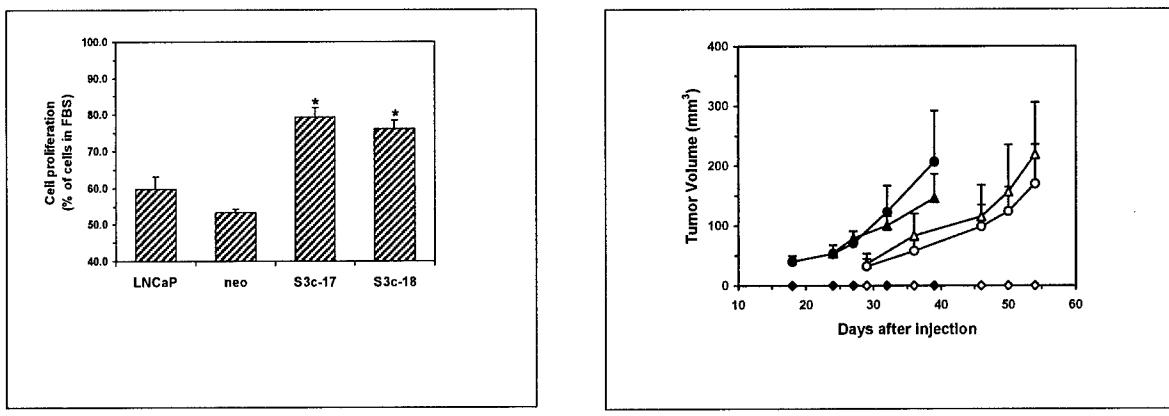


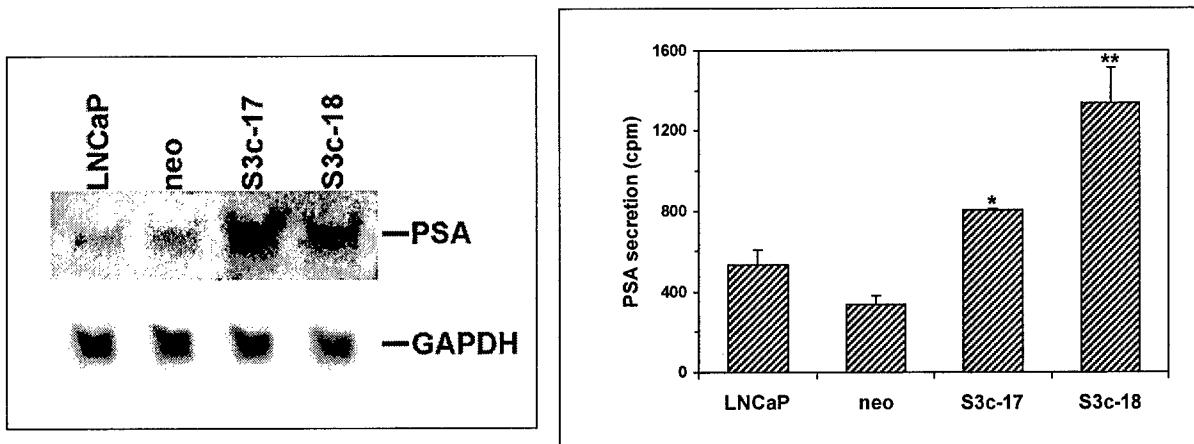
Fig 1. Stat3 DNA-binding activity in LNCaP and LN series cell lines. Whole cell extracts (20  $\mu$ g) were subjected to EMSA using a  $^{32}$ P-labeled oligonucleotide probe containing the consensus binding motif for Stat3.

Task 1B. To examine the effect of Stat3 activation on these cell growth in vitro and in vivo (months 7-12). We demonstrated that cells expressing constitutively activated Stat3 can enhance LNCaP androgen independent growth in vitro as analyzed by MTT assay (Fig 2A) and LNCaP growth in the castrated nude mice (Fig 2B), suggesting that Stat3 can enhance androgen independent growth of androgen-dependent LNCaP cells (Appendix 1).

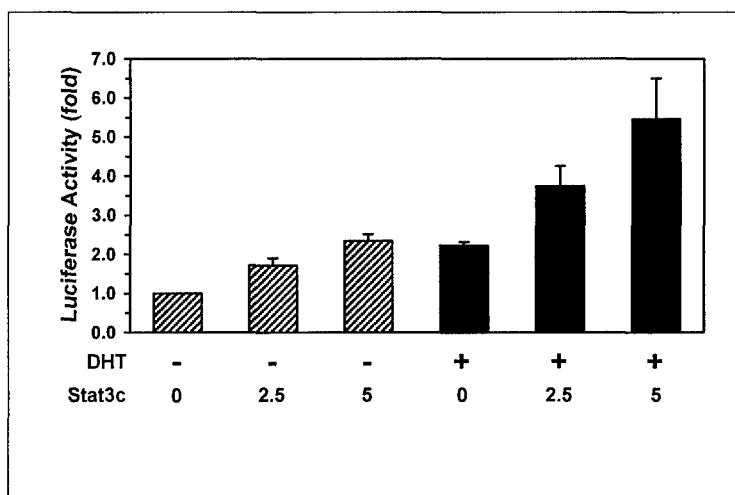
**A****B**

**Figure 2.** Stat3 enhances androgen-independent growth *in vitro*. **(A)** Effect of Stat3 on LNCaP cell growth in the presence and absence of androgen *in vitro*. Cells were cultured in RPMI-1640 supplemented with either 10% FBS or 10% charcoal-stripped FBS. Cell proliferation values in charcoal-stripped FBS were expressed as % relative to the complete FBS. \*,  $P < 0.05$ . **(B)** Stat3 induces androgen-independent growth *in vivo*. Tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone ( $\diamond$ ) or clones that overexpress activated Stat3 (S3c-17,  $\nabla$ ; S3c-18,  $\circ$ ) were injected into the intact (filled symbols) or castrated (open symbols) male nude mice ( $n = 10$  for each condition).

Task 1C. To examine the effect of Stat3 activation on the expression of Stat3 target genes and AR-mediated genes (months 7-18). Prostate specific antigen (PSA) is a typical AR-mediated gene. We demonstrated that Stat3 can enhance PSA express both in mRNA levels as examined by Northern blot (Fig 3A) and in protein levels as examined by ELISA (Fig 3B). In addition, Stat3 can enhance PSA promoter activity and ARE-containing gene transactivation (Fig 4 and appendix 1).



**Figure 3.** Stat3 enhances PSA expression. (A) PSA mRNA expression in Stat3 overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by Northern blot analysis using 20  $\mu$ g of total RNA. GAPDH is a control for equal loading. (B) PSA protein secretion in the absence of androgen. PSA secretion was quantitated by PSA immunoradiometric assay of 50  $\mu$ l of supernatant of cell culture in phenol red-free RPMI containing 10% charcoal-stripped serum. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

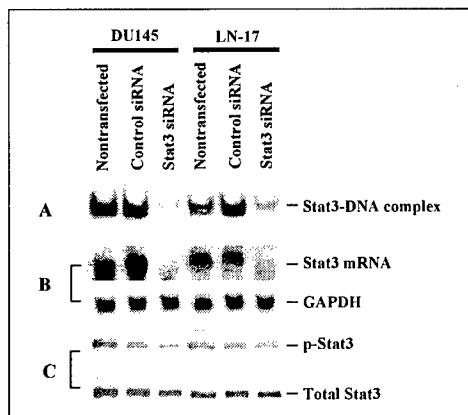


**Figure 4.** (A) Effect of Stat3 on PSA promoter activity in the absence of DHT and in the presence of 10 nM of DHT. LNCaP cells were transiently transfected with PSA-luc reporter, and increasing doses (0, 2.5, 5  $\mu$ g) of Stat3 expression plasmid. Total DNA content was kept constant in all wells. HeLa cells transiently transfected with or without AR expression plasmid, PSA-luc reporter, and increasing doses (0, 2.5, 5  $\mu$ g) of Stat3c expression plasmid in the presence of 10 nM of DHT. Total DNA content was kept constant in all wells. The luciferase activity was measured. Results are displayed as the average of four independent experiments. RLU: relative light units.

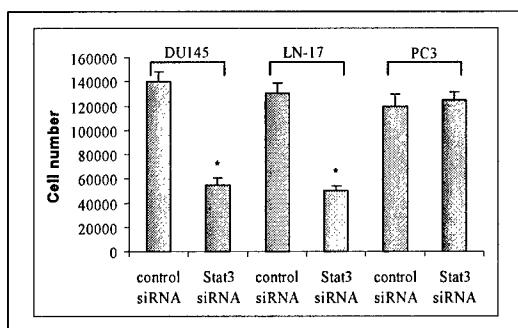
**Task 2. To determine whether Stat3 activation induce androgen-independent growth by affecting AR signaling in prostate cancer cells (months 19-36).**

We are initiating the experiments to study the interaction between Stat3 and AR and the consequences of this interaction in promoting androgen-independent prostate cancer. The experiments were designed to investigate the potential use of RNA interference to block Stat3 expression and activation and the effect on the growth of human prostate cancer cells. We identified a small interfering RNA (siRNA) specific for Stat3 and expressed in human prostate cancer cells from DNA expression vector which employ RNA polymerase III promoters from the U6 small nuclear RNA gene to transcribe siRNAs (Fig. 5). We demonstrate that blockade of Stat3 activation by the Stat3 siRNA suppresses the growth of human prostate cancer cells and Stat3-mediated gene expression and induces apoptotic cell death (Fig. 6, 7). The Stat3 siRNA does not inhibit the proliferation nor induces apoptosis of Stat3-inactive PC3 human prostate cancer cells

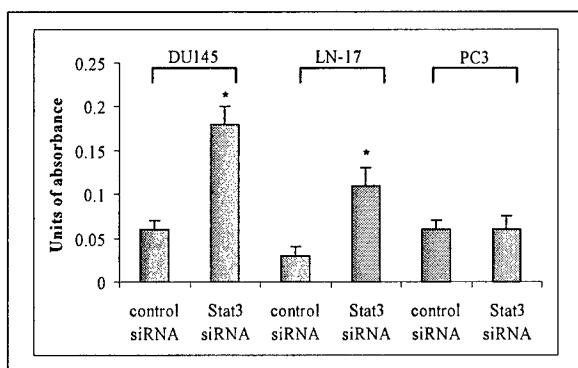
(Fig. 6, 7). In addition, the Stat3 siRNA inhibits the levels of AR-mediated gene- prostate specific antigen (PSA) expression in prostate cancer cells (Fig. 8).



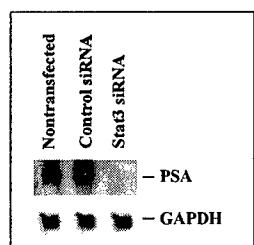
**Figure 5.** Effect of Stat3 siRNA on Stat3 expression and activity of human prostate cancer cells. **A.** Stat3-DNA binding activity. The Stat3 activity was analyzed using EMSA as described in Methods and Materials. **B.** Stat3 mRNA levels of human prostate cancer cells transfected with Stat3 siRNA or control siRNA were determined by Northern blot analysis. GAPDH expression was used as an internal control. **C.** Effect of Stat3 siRNA on Stat3 protein expression. Western blots were performed using either antibodies against phospho-specific Stat3 (Tyr-705) or total Stat3 with whole cell extracts isolated from prostate cancer cells transfected with Stat3 siRNA expression vector or control siRNA vector.



**Figure 6.** Effect of Stat3 siRNA on human prostate cancer cell growth. **A.** Human prostate cancer DU145, PC3 and LN-17 cells ( $1 \times 10^5$  per well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 24 h, the cells were transfected with either Stat3 siRNA expression vector or negative control plasmid. Four days later, the cells were counted with a Coulter counter. Columns represent means of data ( $n=4$ ); bars,  $\pm$  SE. \*, significantly different from control siRNA.



**Figure 7.** Effect of Stat3 siRNA on prostate cancer cell apoptosis. **A.** Cell death analysis by a specific ELISA kit. Human prostate cancer DU145, LN-17, and PC3 cells were transfected with Stat3 siRNA expression vector or control siRNA vector. Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the amount of protein. Data are expressed as mean  $\pm$  SD of four independent experiments. \*, significantly different from control siRNA.

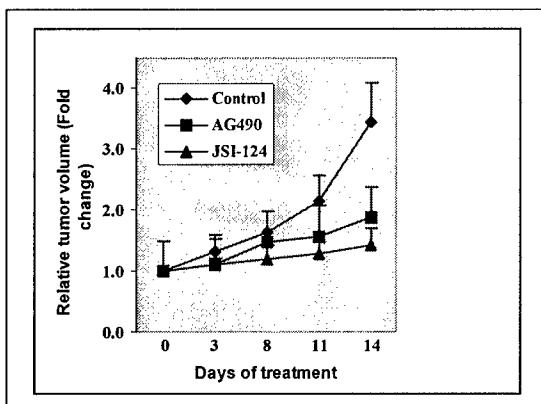


**Figure 8.** Stat3 siRNA inhibits PSA protein expression in LN-17 cells. The levels of PSA protein expression in the medium was analyzed by PSA ELISA from LN-17 cells transfected with either Stat3 siRNA expression vector or control siRNA vector.

In addition, we have examined the potential of tyrosine kinase inhibitors to block Stat3 activation in prostate cancer cells. We evaluated two Stat3 signaling inhibitors: AG490 and JSI-124(Cucurbitacin-I). AG490 is a tyrosine kinase inhibitor selective for the JAK family kinases. We evaluated the effects of AG490 on the growth of prostate cancer cells that express activated Stat3 in vitro and in vivo. We chose to study the following human: LNCaP, DU145, PC3, CWR22Rv1 prostate cell lines. AG490 abrogated Stat3 activity and inhibited the growth of human and prostate cancer cell lines with an  $IC_{50}$  from 20-40  $\mu$ M (Table 1). To study the effects in vivo, we inoculated DU145 cells subcutaneously into the flanks of male nude mice. Once tumors reached a size of  $0.5\text{cm}^3$  they either received AG490 (0.5mg/mice i.p daily for 14 days) or vehicle (DMSO/RPMI) only. AG490 suppressed tumor growth by 50% ( $p<0.01$ ) (Fig. 9). AG490 administration did not cause any toxicity. JSI-124 is a plant natural product. We studied the effects of JSI-124 in human prostate cancer cell lines: LNCaP, DU145, PC3 and CWR22Rv1. We established the  $IC_{50}$  to range 0.5 to 1.0  $\mu$ M (Table 1). Our data suggest that targeting IL-6/Stat3 signaling may represent an opportunity in the development of new treatments for AIPC.

**Table 1.**  $IC_{50}$  ( $\mu$ M) of AG490 and JSI-124 on the growth of prostate cancer cells *in vitro*

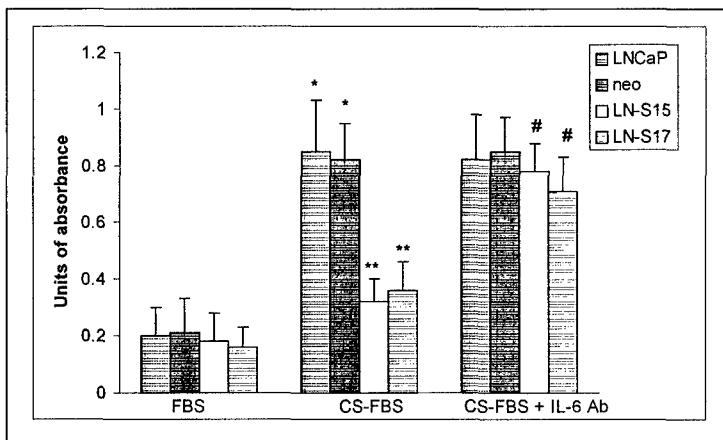
Cell lines	LNCaP	DU145	PC3	CWR22
AG490	40	25	30	30
JSI-124	1	0.5	1	0.5



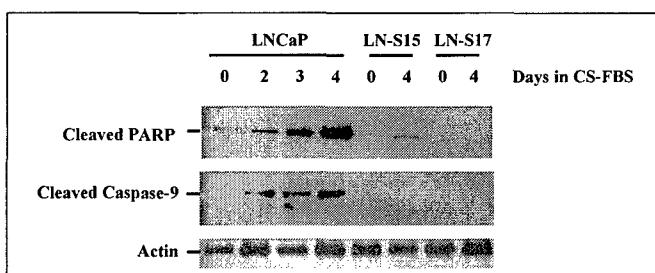
**Figure 9.** AG490 and JSI-124 treatment of DU145 tumor.  $2 \times 10^6$  DU145 cells were injected s.c into nude mice. The mice bearing established tumor were treated with AG490 (0.5 mg/mouse) and JSI-124 (0.1 mg/mouse) for 14 days and tumor volume was measured.

Androgen ablation induces apoptotic death of prostate epithelial cells and is a standard treatment for prostate cancer. However, androgen-independent prostate cancer cells become resistant to apoptosis, rendering androgen ablation therapy ineffective. To understand the role of Stat3 in androgen independent prostate cancer, we investigated the role of Stat3 activation in IL-6-mediated antiapoptotic activity in prostate cancer cells. We demonstrate that overexpression of IL-6 renders androgen sensitive LNCaP human

prostate cancer cells more resistant to apoptosis induced by androgen deprivation (Fig 10). LNCaP cells undergo apoptosis after 72 h of androgen deprivation, an outcome is largely absent in clones overexpressing IL-6 as measured by cell death ELISA (Fig 10). IL-6 over-expressing cells resulted in a significant decrease in the expression of pro-apoptotic proteins such as cleaved PARP and cleaved caspase-9 as well as an increase in the expression of anti-apoptotic proteins Bcl-x<sub>L</sub> and phosphorylated Bad (Fig 11). Addition of IL-6 antibody completely abolished the anti-apoptotic activity of IL-6 (Fig 11B). This protective effect of IL-6 was reversed by the expression of a dominant-negative Stat3 mutant, Stat3F (Fig. 12). Furthermore, ectopic expression of a constitutively active Stat3 antagonized androgen deprivation-induced cell death of LNCaP cells (Fig. 13). These results indicate that IL-6 protects androgen sensitive LNCaP cells from apoptosis induced by androgen deprivation, and Stat3 activation play an important role in IL-6-mediated anti-apoptosis in prostate cancer cells.



compared with neo controls in CS-FBS conditions; #,  $p < 0.01$  compared with LN-S15 and LN-S17 in the CS-FBS condition in the absence of the IL-6 antibody.



FBS conditions for 3 days as indicated. A. Immunoblots were analyzed with cleaved PARP and cleaved caspase-9 antibodies.

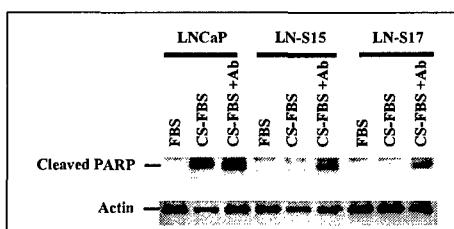
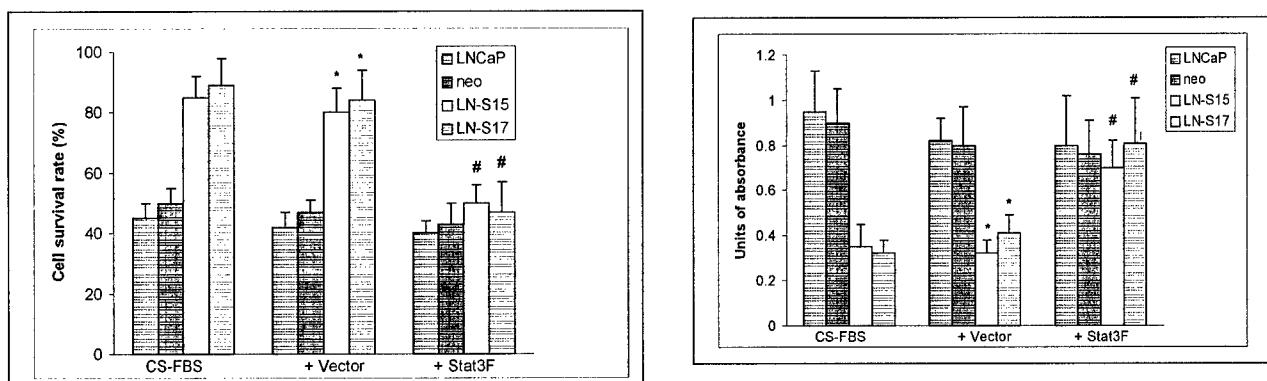
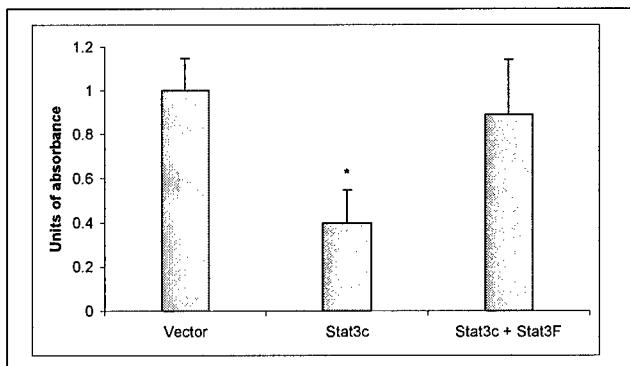


Fig 11 B. Twenty  $\mu$ g/ml of IL-6 antibody were added to the androgen deprived charcoal stripped culture medium (CS-FBS-Ab), and whole cell lysate were immunoblotted with cleaved PARP antibody. C. Immunoblots were analyzed with Bcl-x<sub>L</sub> and phospho-Bad antibodies.



**Figure 12.** Stat3F inhibits anti-apoptotic activity of IL-6. **Left.** Stat3F inhibits IL-6 induced LNCaP cell survival in androgen deprived media. Parental LNCaP, neo, IL-6 overexpressing LN-S15 and LN-S17 cells were cultured in RPMI 1640 supplemented with 10% charcoal-stripped FBS (CS-FBS). The cells were then transiently transfected with equal amounts (2  $\mu$ g) of either vector controls or Stat3F. The cell numbers were counted after 3 days in CS-FBS condition and cell survival values were expressed as % relative to the complete FBS. **Right.** Stat3F blocks anti-apoptotic activity of IL-6 in androgen deprived conditions. Parental LNCaP, neo, IL-6 overexpressing LN-S15 and LN-S17 cells were cultured in RPMI 1640 supplemented with 10% charcoal-stripped FBS (CS-FBS). The cells were then transiently transfected with equal amounts (2  $\mu$ g) of either vector controls or Stat3F. Quantitation of apoptosis by a special ELISA kit in these cells after cultured in androgen deprived CS-FBS for 3 days. Data are expressed as mean  $\pm$  S.E. of four independent experiments. \*,  $P < 0.05$  compared with neo control in CS-FBS conditions; #,  $p < 0.05$  compared with LN-S15 and LN-S17 transfected with vector control in the CS-FBS condition.



**Figure 13.** Constitutively activated Stat3 protects LNCaP cells apoptosis induced by androgen deprivation. LNCaP cells were cultured in RPMI 1640 supplemented with 10% charcoal-stripped FBS (CS-FBS). The cells were then transiently transfected with equal amounts (2  $\mu$ g) of either vector controls or constitutively activated Stat3c or Stat3c plus Stat3F. Quantitation of apoptosis by a special ELISA kit was performed after cultured in androgen deprived CS-FBS for another 3 days.

Data are expressed as mean  $\pm$  S.E. of four independent experiments. \*,  $P < 0.05$  compared with vector control in CS-FBS conditions.

### Key research accomplishments

- We demonstrated that Stat3 plays a critical role in prostate cancer growth.
- Stat3 enhances AR-mediated gene expression such as PSA.
- Stat3 enhances androgen independent growth of prostate cancer cells *in vitro* and *in vivo*.
- Stat3 activates androgen receptor (AR) in the presence and in the absence of androgen.

- Stat3 activation is required for IL-6 mediated antiapoptotic activity in prostate cancer cells.
- Targeting Stat3 signaling by siRNA or tyrosine kinase inhibitors inhibits growth and induces apoptosis of prostate cancer cells.

### Reportable outcome

#### Publications:

1. Lee SO, Lou W, Qureshi KM, Mehraein-Ghomi F, Trump DL, and **Gao AC**. RNA interference targeting Stat3 inhibits growth and induces apoptosis of human prostate cancer cells. *Prostate*, 60(4):303-9, 2004.
2. Lee SO, Lou W, Johnson CS, Trump DL, and **Gao AC**. Interleukin-6 protects LNCaP cells from apoptosis induced by androgen deprivation through the Stat3 pathway. *Prostate* 60: 178-186 , 2004.
3. Lee S.O., Lou W., Hou, M., Onate S.A., and **Gao A.C.** Interleukin-4 enhances prostate specific antigen expression by activation of the androgen receptor and Akt pathway. *Oncogene* 22 (39), 6037-6044, 2003.
4. Lee SO, Lou W, Demiguel F, Hou M, **Gao AC**. Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. *Clin. Cancer Res.*, 9: 370-376, 2003.
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### Conclusions

We demonstrated that activation of Stat3 in androgen-sensitive LNCaP prostate cancer cells results in enhancement of tumor growth in both intact and castrated male nude mice, and enhances androgen receptor-mediated prostate specific antigen (PSA) expression. These findings demonstrate that intracellular signaling mediated by Stat3 can enhance the growth of androgen-sensitive human LNCaP prostate cancer cells in both intact and castrated male nude mice. Targeting Stat3 signaling by siRNA may serve as a novel therapeutic approach for prostate cancer.

### **References and Appendices**

- DeMigeul F., Lou W., Lee S. O., Xiao X., Pflug B., Nelson J.B., and **Gao A.C.** Stat3 enhances the growth of LNCaP human prostate cancer cells in intact and castrated male nude mice. *The Prostate*, 52: 123-129, 2002.
- Lee SO, Lou W, Qureshi KM, Mehraein-Ghom F, Trump DL, and **Gao AC**. RNA interference targeting Stat3 inhibits growth and induces apoptosis of human prostate cancer cells. *Prostate*, 60(4):303-9, 2004.
- Lee SO, Lou W, Johnson CS, Trump DL, and **Gao AC**. Interleukin-6 protects LNCaP cells from apoptosis induced by androgen deprivation through the Stat3 pathway. *Prostate* 60: 178-186 , 2004.
- Lee SO, Lou W, Demiguel F, Hou M, **Gao AC**. Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. *Clin. Cancer Res.*, 9: 370-376, 2003.

## Stat3 Enhances the Growth of LNCaP Human Prostate Cancer Cells in Intact and Castrated Male Nude Mice

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**BACKGROUND.** Prostate cancer frequently progresses from an initial androgen dependence to androgen independence, rendering the only effective androgen ablation therapy useless. The mechanism underlying the androgen-independent progression is unknown. Stat3, a member of the family of signal transducers and activators of transcription, is activated in numerous cancers, including prostate. This study is to investigate the role of Stat3 activation in the growth of prostate cancer cells.

**METHODS.** A constitutively active Stat3 was ectopically expressed in androgen-sensitive LNCaP prostate cancer cells and resulting stable clones expressing activated Stat3 were isolated. The effect of Stat3 activation on LNCaP cell growth in response to androgen in vitro and in vivo was examined.

**RESULTS.** We show that the levels of activated Stat3 are associated with the progression of androgen-independent prostate cancer. Activation of Stat3 in androgen-sensitive LNCaP prostate cancer cells results in enhancement of tumor growth in both intact and castrated male nude mice and enhances androgen receptor-mediated prostate specific antigen expression.

**CONCLUSIONS.** These findings demonstrate that intracellular signaling mediated by Stat3 can enhance the growth of androgen-sensitive human LNCaP prostate cancer cells in both intact and castrated male nude mice. *Prostate* 52: 123–129, 2002. © 2002 Wiley-Liss, Inc.

**KEY WORDS:** Stat3; androgen-independence; androgen receptor; prostate cancer

### INTRODUCTION

Hormone-refractory prostate cancer refers to a resistance to androgen ablation therapy, the only effective systemic therapy available for advanced prostate cancer. Almost all patients with advanced prostate cancer respond initially to androgen ablation therapy. However, virtually every patient will relapse to hormone-refractory disease due to the growth of androgen-independent cancer cells. There is growing evidence supporting the concept that the paracrine and autocrine loops mediated by growth factors and cytokines play an important role in acquisition of hormone independence [1,2]. Stat3, a member of Janus Kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) signaling pathway, is implicated in many cytokine-, hormone-, and growth factor-

mediated signaling pathways to regulate a variety of biological responses, including development, differentiation, cell proliferation, and survival [3,4].

Constitutively activated Stat3 protein is found in various types of tumors, including leukemia, breast,

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head and neck, and prostate [5–10]. In addition, constitutively activated Stat3 (a mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3) induces cellular transformation and tumor formation in nude mice [11]. These results suggest that Stat3 may function as an oncogene and play a critical role in transformation and tumor progression. Here, we investigated the effect of Stat3 activation on the growth of androgen-sensitive LNCaP cells in the intact and castrated male nude mice.

## MATERIALS AND METHODS

### Cell Culture and Plasmids

Human LNCaP prostate cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The LNCaP cells were passaged twice weekly, and the passage number approximately eight were used for all of the studies. Androgen-insensitive LN95, LN96, LN97, and LN98 human prostate cancer cells [12] were maintained in phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped serum (Hyclone, CA). HeLa cells were routinely maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% FBS. The cells were grown at 37°C in 5% CO<sub>2</sub> and 95% air. The plasmid (pSG5-wtAR) containing the wild-type full-length sequence of human androgen receptor (AR) was kindly provided by Dr. Chawnshang Chang, University of Rochester, NY. The plasmid containing the constitutively active form of Stat3c was provided by Dr. James Darnell, Jr., The Rockefeller University, NY [11]. Prostate specific antigen (PSA) regulatory element containing an 822-bp enhancer [13] and a 620-bp promoter [14] was generated by PCR amplification of Hirt DNA from LNCaP cells by using primers for the enhancer (5' primer, 5'-GCGGTACCCTGCAGAGAAT; and 3' primer, 5'-GGATCCCCATGGTTCTGTC), and for the promoter (5' primer, GGATCCTTGGATTTGAAAT; and 3' primer, 5'-GGTCTAGAAAGCTGGGGCT). The PCR products of the amplified enhancer and promoter were gel purified, kinased, and inserted into the *Eco*RV site of pBluescript KS + vector, separately. The PSA regulatory element was generated by inserting the enhancer (cut out with *Bam*HI from the pBluescript KS + construct) in front of the promoter at the *Bam*HI site of the pBluescript construct containing the promoter. The PSA reporter construct (pAAV-PSA-Luc) was generated by insertion of the 1.4-kb fragment of the PSA enhancer and promoter in place of the CMV promoter of the pAAV-CMV-Luc construct [15].

### Luciferase Assay

Twenty-four hours before transfection,  $3 \times 10^5$  cells were plated in a six-well plate in phenol red-free DMEM containing 5% dextran-coated charcoal-stripped FBS (CS-FBS). Cells were transfected with a total amount of 5  $\mu$ g of DNA by using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. The total amount of plasmid DNA used was normalized to 5  $\mu$ g/well by the addition of empty plasmid. Three hours later, the DNA:liposomes mixture was removed and cells were treated with phenol red-free medium containing 5% CS-FBS with either 10 nM dehydrotestosterone (DHT, Sigma, St. Louis, MO) or in the absence of DHT. Cell extracts were obtained 36 hr later, and luciferase activity was assayed by using the Luciferase Assay System (Promega, Madison, WI). Protein concentration in cell extracts was determined by Coomassie Plus protein assay (Pierce, Rockford, IL). Luciferase activities were normalized by protein concentrations of the samples. All transfection experiments were performed in triplicate wells and repeated at least four times.

### Transfection and Northern Blot

Transfections with the plasmid expressing the constitutively active form of Stat3c or empty vector were performed by using Superfect (Qiagen, Valencia, CA) according to the manufacturer's protocol. Stable clones were selected in 800  $\mu$ g/ml G418 and maintained in 300  $\mu$ g/ml G418.

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, Rockville, MD). Twenty micrograms of each sample was electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 1.2-kb *Bam*HI fragment of the PSA cDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) by using the Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out during 3 hr at 65°C in Rapid-hyb buffer (Amersham). Membranes were washed for 15 min at 65°C in 2  $\times$  SSC, 0.1% sodium dodecyl sulfate (SDS; twice), 0.5  $\times$  SSC, 0.1% SDS and 0.1  $\times$  SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio-Rad, Hercules, CA).

### Electromobility Shift Assay

Whole cell extracts were prepared and electromobility shift assays (EMSA) were performed as described previously [8]. For supershift analyses, the cell extracts were preincubated with antibody specifically

against Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA). The protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel in 1×TBE (90 mM Tris-borate, 2 mM EDTA) at room temperature, and the results were analyzed as above.

### Western Blot

Whole cell extracts were obtained by lysing the cells in RIPA buffer (1× phosphate buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, with freshly added protease inhibitors: 0.1 mM phenylmethyl sulfonyl fluoride, 1 mM sodium orthovanadate, 30 µl/ml aprotinin). Whole cell extracts were resolved in 12.5% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% milk in PBS-0.1% Tween 20, membranes were incubated for 1 hr at room temperature with anti-AR rabbit polyclonal antibody (Santa Cruz Biotechnology) or anti-FLAG antibody (Sigma), or anti-phospho-Stat3 antibody (Cell Signaling Technology, MA) diluted in 1% milk in PBS-Tween. After secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

### In Vivo Assays

The mice were injected in the flank with  $3 \times 10^6$  cells resuspended in Matrigel diluted 1:1 in complete culture medium. The volume of the growing tumors was estimated by measuring their three dimensions (length × width × depth) with a caliper.

### PSA Protein Analysis

PSA secretion was quantitated by PSA immunoradiometric assay (Beckman Coulter, Fullerton, CA) of tissue culture supernatant. Equal numbers of cells were plated in phenol red-free RPMI containing 10% FBS. Cells were allowed to attach for 24 hr, then the medium was changed to phenol red-free medium supplemented with 10% charcoal-stripped serum. After another 2 days, 50 µl of supernatant was assayed for PSA.

### Statistical Analysis

Values were expressed as the mean  $\pm$  SE. Statistical analyses were performed by one-way analysis of variance, followed by the Student-Newman-Keuls test for multiple comparisons, with a  $P < 0.05$  being considered significant.

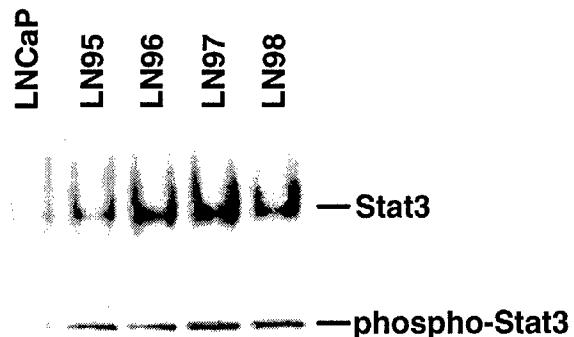
## RESULTS AND DISCUSSION

### Stat3 Activation Is Associated With Androgen-Independent Progression

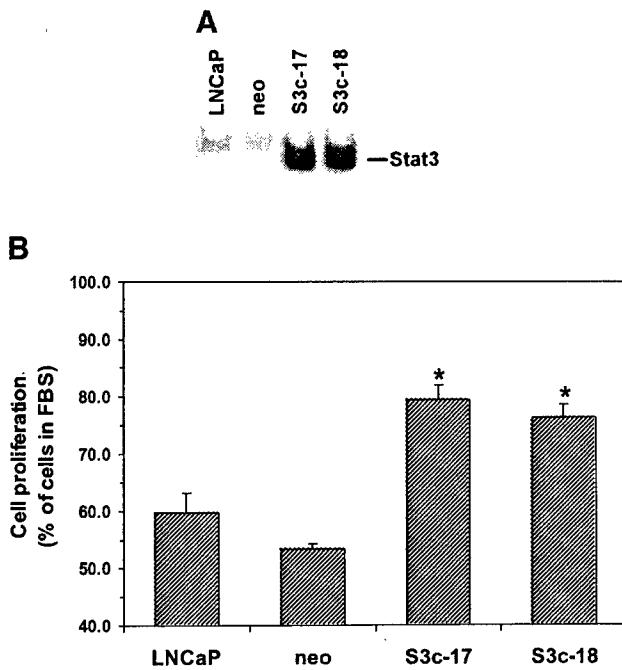
We first tested whether Stat3 activation is associated with the progression of androgen-independent prostate cancer. We analyzed Stat3 activity by EMSA and the expression of phosphorylated Stat3 protein in androgen-sensitive LNCaP human prostate cancer cells and androgen-insensitive sublines (LN95, LN96, LN97, and LN98) derived from LNCaP cells [12]. LNCaP sublines (LN95, LN96, LN97, and LN98) were derived from LNCaP cells after chronic androgen deprivation in vitro, which produces androgen-insensitive clones [12]. All of the LNCaP sublines retain the AR and produce PSA even in the absence of androgen [12]. The androgen-insensitive LNCaP sublines grew readily in both castrated and intact male nude mice compared with no tumors formed in wild-type LNCaP animals subcutaneously [12]. Both the levels of Stat3 activity and phosphorylated Stat3 protein were increased in the androgen-insensitive sublines compared with that of the parental LNCaP cell line (Fig. 1). These results suggest that androgen-insensitive growth is associated with increased levels of Stat3 activity in LNCaP human prostate cancer model.

### Stat3 Activation Enhances LNCaP Cell Growth In Vitro and In Vivo

Because LNCaP cells have minimal levels of Stat3 activity, we tried to determine whether elevation of



**Fig. 1.** Stat3 activation is associated with androgen-independence. **Top panel:** Stat3 DNA-binding activity in LNCaP and LN series cell lines. Whole cell extracts (20 µg) were subjected to EMSA by using a  $^{32}$ P-labeled oligonucleotide probe containing the consensus binding motif for Stat3. **Bottom panel:** Stat3 tyrosine phosphorylation (phospho-Stat3) in LNCaP and LN series cell lines. Forty micrograms of whole cell protein extract was analyzed by Western blot by using antibodies specific against phosphotyrosine Stat3 (Tyr-705) antibody.



**Fig. 2.** Stat3 enhances androgen-independent growth in vitro. **A:** Stat3 DNA binding activity in Stat3-overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by EMSA. **B:** Effect of Stat3 on LNCaP cell growth in the presence and absence of androgen in vitro. Cells were cultured in RPMI-1640 supplemented with either 10% FBS or 10% charcoal-stripped FBS. Cell proliferation values in charcoal-stripped FBS were expressed as percentage relative to the complete FBS. \* $P < 0.05$ .

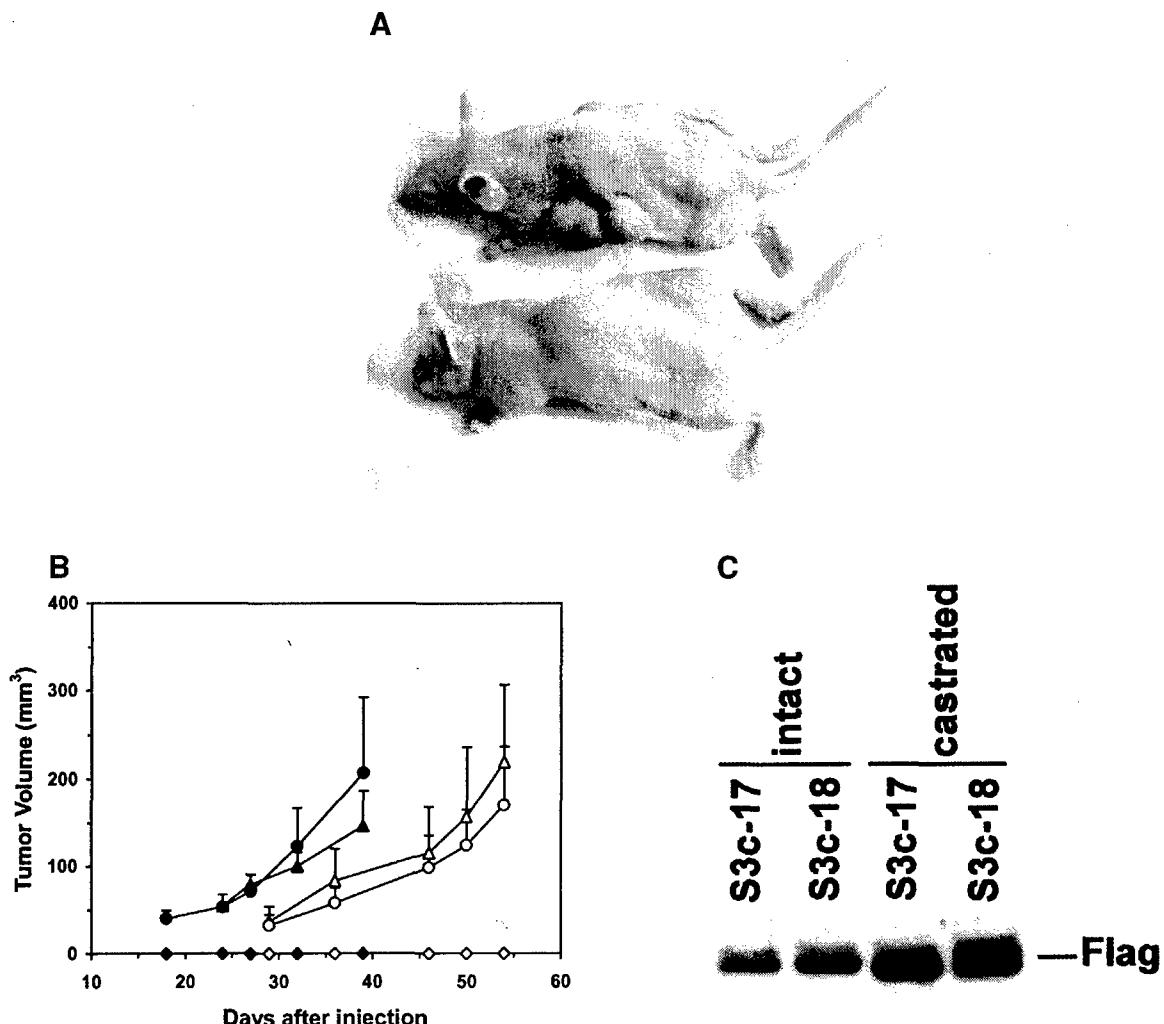
Stat3 activity will enhance LNCaP cell growth in vitro and in vivo. We introduced a constitutively activated Stat3 into the androgen-sensitive LNCaP cells. Two independent clones overexpressing activated Stat3 were selected (Fig. 2A). The growth of these cells in normal serum and in androgen-deprived serum was compared. The growth of LNCaP cells and neo vector alone control cells in culture was reduced by approximately 50% after 48 hr in androgen-deprived charcoal-stripped serum compared with that in the normal serum (Fig. 2B). In both clones of LNCaP cells overexpressing Stat3, however, there was only a 20% decrease in growth under these androgen-deprived conditions compared with growth in normal serum (Fig. 2B), suggesting that activated Stat3 can enhance the growth of LNCaP cells in the absence of androgen in vitro.

LNCaP is a human prostate cancer cell line derived from supraclavicular lymph node metastases [16,17]. These cells express mutant but functional androgen receptors and exhibit androgen-sensitive phenotype

[16,17]. Igawa et al. reported that the aggressiveness and androgen responsiveness of LNCaP cells can be altered by culturing the cells continuously in vitro [18]. LNCaP cells in the early passages are usually less aggressive (i.e., lower tumorigenic in vivo) and higher androgen responsiveness in vitro and in vivo than that of the cells in their late passages [18]. The parental LNCaP cells used in the present study are in their early passages (see Materials and Methods section). The parental LNCaP cells and vector control Neo clone did not grow any detectable tumor in both intact (within 40-day observation period) and castrated (within 60-day observation period) male mice (Fig. 3B). We next tested the effect of Stat3 activation on LNCaP tumor growth in both intact and castrated male nude mice. Eight-week-old male nude mice were randomly divided into two groups, one left intact, another group received surgical castration. Three days after castration, intact or castrated male nude mice were injected subcutaneously with LNCaP cell clones overexpressing Stat3. For the two independent Stat3-overexpressing clones, tumors became apparent at the site of injection within 20 days in the intact male mice and within 30 days in the castrated male mice (Fig. 3A). There was a delay of the latency for tumor formation in the castrated male mice compared with the intact male mice (Fig. 3B). Western blot analysis of protein extracts derived from Stat3-overexpressing tumors in both intact and castrated male mice revealed high levels of FLAG-tagged Stat3 protein (Fig. 3C), indicating that the growing tumor cells continue to express FLAG-tagged Stat3 plasmids and are derived from human LNCaP cells. These results demonstrate that Stat3 activation not only enhances prostate cancer cell tumor growth in vivo, but also promotes tumor growth in the androgen-deprived castrated male nude mice.

#### Stat3 Enhances AR-Mediated Gene Expression Independent of Androgen

To determine whether Stat3 activation affects AR signaling, we tested the effects of Stat3 on the expression of endogenous PSA, a well-characterized prostate specific antigen whose transcription is strictly regulated by androgen [19]. The levels of PSA mRNA expression from LNCaP cell clones overexpressing activated Stat3 were increased compared with the parental LNCaP cells (Fig. 4A). To examine the effects of Stat3 activation on the expression of PSA upon androgen withdrawal, we measured PSA protein secretion in phenol red-free medium supplemented with the charcoal-treated serum. As shown in Figure 4B, the levels of PSA protein expression were increased in the Stat3-overexpressing LNCaP

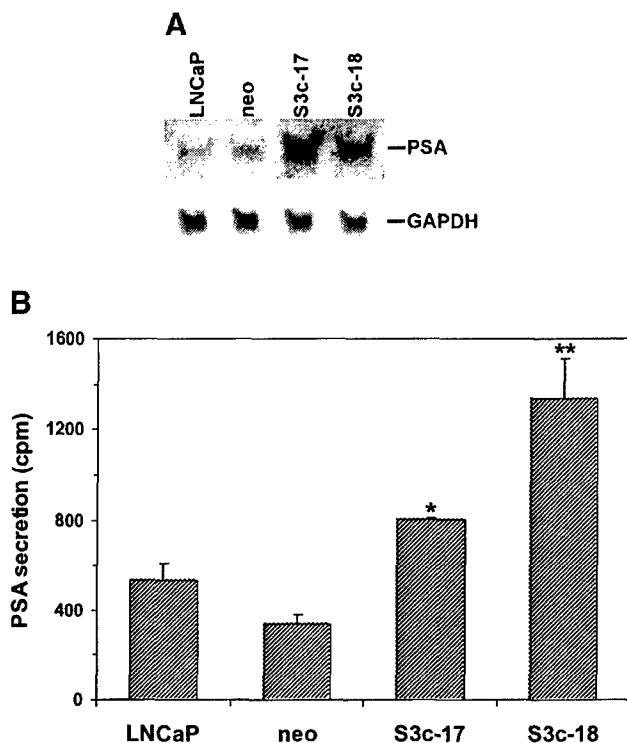


**Fig. 3.** Stat3 induces androgen-independent growth in vivo. **A:** Stat3-overexpressing clone (S3c-18) developed tumors vs. parental LNCaP cells, which did not grow any tumors in the castrated male nude mice. **B:** Tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone ( $\diamond$ ) or clones that overexpress activated Stat3 (S3c-17, triangles; S3c-18, circles) were injected into the intact (filled symbols) or castrated (open symbols) male nude mice ( $n = 10$  for each condition). **C:** Western blot analysis of Flag-tag expression in cell extracts from Stat3-derived tumors in the intact and castrated male nude mice.

subclones compared with the parental LNCaP cells and vector controls in androgen-deprived conditions, indicating that Stat3 can partially replace androgen function in activation of the AR-mediated PSA gene expression. Tumors expressing Stat3 also produced high levels of circulating PSA in the serum (average, 32 ng/ml per gram of tumor) in the castrated male mice.

To determine the effect of Stat3 activation on AR-mediated gene transcription, we transiently transfected LNCaP cells with a luciferase reporter linked to the androgen-responsive promoter of PSA and

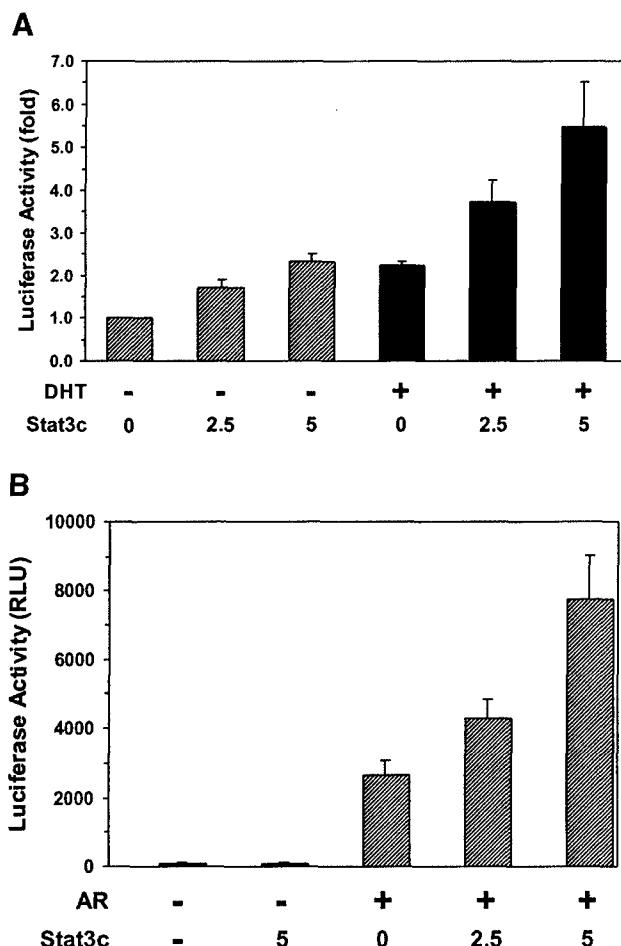
various amounts of expression vectors encoding the constitutively active Stat3 [11]. To compare the effect of Stat3 on PSA promoter activity in the presence and in the absence of the androgen, the cells were then cultured in phenol red-free medium supplemented with the charcoal-stripped serum either in the presence of 10 nM of dehydrotestosterone (DHT) or in the absence of DHT. After 24 hr, cells were harvested and luciferase activities were determined. As shown in Figure 5A, Stat3 activated the PSA-luc reporter in a concentration-dependent manner in the absence of androgen, suggesting that Stat3 activates PSA tran-



**Fig. 4.** Stat3 enhances prostate specific antigen (PSA) expression. **A:** PSA mRNA expression in Stat3-overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by Northern blot analysis by using 20  $\mu$ g of total RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a control for equal loading. **B:** PSA protein secretion in the absence of androgen. PSA secretion was quantitated by PSA immunoradiometric assay of 50  $\mu$ l of supernatant of cell culture in phenol red-free RPMI containing 10% charcoal-stripped serum. \* $P < 0.05$ ; \*\* $P < 0.01$ .

scription in a ligand-independent manner. Addition of 10 nM of DHT-enhanced PSA-luc reporter activity induced by Stat3 (Fig. 5A). To test whether the effects of Stat3 on PSA transcription require AR, we transiently transfected AR-negative HeLa cells [20], with or without an AR expression vector, plus a luciferase reporter with the androgen-responsive promoter of PSA (PSA-Luc), and increasing amounts of expression vector encoding the constitutively active Stat3c. Cotransfections lacking the AR failed to result in activation of PSA-luc reporter gene (Fig. 5B), suggesting that the effects of Stat3 on androgen responsive gene expression are dependent on AR.

Our findings demonstrate that increased Stat3 activity promotes tumor growth of androgen-sensitive LNCaP prostate cancer cells in both intact and castrated male nude mice and enhances AR-mediated



**Fig. 5.** **A:** Effect of Stat3 on prostate specific antigen (PSA) promoter activity in the absence of dehydrotestosterone (DHT) and in the presence of 10 nM of DHT. LNCaP cells were transiently transfected with PSA-luc reporter and increasing doses (0, 2.5, 5  $\mu$ g) of Stat3 expression plasmid. Total DNA content was kept constant in all wells. **B:** The effect of Stat3 on PSA transcription requires androgen receptor (AR). HeLa cells transiently transfected with or without AR expression plasmid, PSA-luc reporter, and increasing doses (0, 2.5, 5  $\mu$ g) of Stat3c expression plasmid in the presence of 10 nM of DHT. Total DNA content was kept constant in all wells. The luciferase activity was measured. Results are displayed as the average of four independent experiments. RLU, relative light units.

PSA expression both in the presence and absence of androgen.

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## RNA Interference Targeting Stat3 Inhibits Growth and Induces Apoptosis of Human Prostate Cancer Cells

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Stat3, a member of the signal transduction and activation of transcription (STAT) family, is a key signal transduction protein that mediates signaling by cytokines, peptide growth factors, and oncoproteins and is constitutively activated in numerous cancers including prostate. Previous studies demonstrated that constitutively activated Stat3 plays an important role in the development and progression of prostate cancer by promoting cell proliferation and protecting against apoptosis. The present study was designed to investigate the potential use of RNA interference to block Stat3 expression and activation and the effect on the growth of human prostate cancer cells. We identified a small interfering RNA (siRNA) specific for Stat3 and demonstrate that blockade of Stat3 activation by the Stat3 siRNA suppresses the growth of human prostate cancer cells and Stat3-mediated gene expression and induces apoptotic cell death. The Stat3 siRNA does not inhibit the proliferation nor induces apoptosis of Stat3-inactive human prostate cancer cells. In addition, the Stat3 siRNA inhibits the levels of androgen-regulated prostate specific antigen (PSA) expression in prostate cancer cells. These results demonstrate that targeting Stat3 signaling using siRNA technique may serve as a novel therapeutic strategy for treatment of prostate cancer expressing constitutively activated Stat3.

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**KEY WORDS:** Stat3; RNA interference; prostate cancer; apoptosis

### INTRODUCTION

Stat3, a member of the signal transduction and activation of transcription (STAT) family, is a key signal transduction protein that mediates signaling by numerous cytokines, peptide growth factors, and oncoproteins [1]. Accumulating evidence demonstrates that Stat3 activation plays important roles in cell differentiation, proliferation, development, apoptosis, and inflammation [2]. Elevated activity of Stat3 has been found frequently in a wide variety of human tumors, including hematologic malignancies, head and neck, breast and prostate cancer [2]. Cell lines from multiple myelomas that have become growth factor independent require constitutively active Stat3 to protect against apoptosis [3]. In addition, constitutively activated Stat3 induces cellular transformation in vitro and tumor formation in nude mice [4].

Studies to date provide strong evidence that aberrant Stat3 signaling plays an important role in the development and progression of prostate cancer by

promoting cell proliferation and protecting against apoptosis. Stat3 activity is not only significantly increased in human primary prostate cancer tissues as compared with normal, but it is also increased in androgen independent prostate cancer cells as compared to androgen sensitive cells [5,6]. Stat3 is a major mediator of interleukin-6 (IL-6) induced signaling in prostate cancer cells and that IL-6 induced androgen

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receptor (AR)-mediated gene activation requires the activation of Stat3 in LNCaP prostate cancer cells [7,8]. Constitutive activation of Stat3 promotes androgen independent growth of androgen dependent LNCaP cells in vitro and in vivo [9]. Blockade of Stat3 expression in human prostate cancer cells suppress proliferation in vitro and tumorigenicity in vivo [10]. These studies demonstrate that constitutively activated Stat3 is not only associated with prostate cancer, but also induces prostate cancer cell proliferation. Thus, the Stat3 signaling pathway may represent a new molecular target for novel therapeutic approaches for prostate cancer. Strategies that target Stat3 signaling have been proposed including tyrosine kinase inhibitors such as tyrphostin AG490 and cucurbitacin I [11,12], antisense oligonucleotides and decoy oligonucleotides [6,13], and dominant-negative Stat3 protein [10,14].

Small interfering RNA (siRNAs) are short double-stranded RNA molecules that can target complementary mRNAs for degradation via a cellular process termed RNA interference (RNAi) [15]. RNAi is usually activated by introducing long double-stranded RNA molecules into cells which are cleaved into 21- to 23-nt RNAs referred to as siRNAs by an endonuclease named Dicer in animal cells [15]. The siRNA molecules then serve as a guide for sequence-specific degradation of homologous mRNAs. SiRNA has been used for functional analysis of genes in many species including invertebrates, plants, and mammalian cells [16]. Recently, siRNA has emerged as powerful RNAi reagents for directed posttranscriptional gene silencing and inhibition of viral propagation [17]. The potential of using siRNA for silencing specific genes has been demonstrated in treatment of viral diseases and cancer including HIV, human papillomavirus, and hepatitis C virus [18].

The present study was designed to investigate the potential use of siRNA to block Stat3 expression and the effect on growth of human prostate cancer cells. We identified a siRNA specific for Stat3 and expressed in prostate cancer cells. We demonstrate that blockade of Stat3 expression by this siRNA inhibits the growth of human prostate cancer cells and induces apoptotic cell death. These results demonstrate that targeting Stat3 signaling using siRNA technique may serve as a novel therapeutic strategy for treatment of prostate cancer expressing constitutively activated Stat3.

## MATERIALS AND METHODS

### Tissue Culture

Human DU145 prostate cancer cells were maintained in RPMI1640 supplemented with 10% of FBS. LN-17 cells were generated from human LNCaP prostate cancer cells stably expressing IL-6 as described

previously [8,19], and cultured in RPMI 1640 supplemented with 10% FBS plus 0.3 mg/ml of G418. Human PC3 prostate cancer cells were maintained in DMEM supplemented with 10% FBS. The cells were cultured in 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C in 5% CO<sub>2</sub> incubator.

### Plasmids and siRNA Expression

The oligonucleotides containing sequences specific for Stat3 (5'-GATCCCGTGTCTATCAGCACAA-  
TTCAAGAGAATTGTGCTGATAGAGAACATT-  
TTGGAAA-3' and 5'-AGCTTTCCAAAAATGTC-  
TCTATCAGCACAAATTCTCTGAAATTGTGCTGA-  
TAGAGAACACGG-3') were synthesized and annealed. Stat3 siRNA expression vector that express hairpin siRNAs under the control of the mouse U6 promoter was constructed by inserting pairs of the annealed DNA oligonucleotides into the pSilencer-neo siRNA expression vector that was digested with BamHI and HindIII (Ambion, Austin, TX). A pSilencer neo vector that expresses a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes was used as a negative control (Ambion). The negative control siRNAs have been tested in multiple cell lines and they show no toxicity to cells when analyzed by Trypan Blue staining and cell counting 48 hr after transfection. The negative control siRNAs have no effect on the expression of the mRNA levels of "housekeeping" genes, including 28S rRNA, GAPDH, and Cyclophilin (Ambion).

### Stat3 siRNA Transfection

DU145, PC3, and LN-17 cells were transiently transfected either with Stat3 siRNA or the negative control plasmid using SuperFect Transfection Reagent (QIAGEN, Inc., Valencia, CA). Briefly, cells were plated into either 12-well plates (10<sup>5</sup> cells per well) or 100 mm dish (10<sup>6</sup> cells) and allowed to adhere for 24 hr. Cells were transfected with either Stat3 siRNA expression vector or negative control siRNA plasmids in serum-free medium for 4 hr, incubated with complete medium for 24 hr, and then cells were switched into medium containing G418 (0.3 mg/ml). Two days later, protein extracts and total RNA were prepared for subsequent analysis.

### In Vitro Growth Assay

DU145, PC3, and LN-17 cells (1 × 10<sup>5</sup> per well) were plated in 12-well plates in RPMI containing 10% FBS. Cells were transfected with either Stat3 siRNA expression vector or negative control plasmid as described above. Four days later, cells were counted with a Coulter counter.

### Apoptosis ELISA Test

Cell death detection ELISA kit (catalog # 1544675) was obtained from Roche Molecular Biochemicals (Indianapolis, IN) and the protocol was followed according to the manufacturer's instructions. For the preparation of samples, cells were plated and transfected with either 1  $\mu$ g of Stat3 siRNA expression vector or 1  $\mu$ g of control plasmid as described previously. Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the amount of protein. The protein concentration was determined by Bradford assay (Coomassie Plus, Pierce, Rockford, IL).

### Luciferase Assay

DU145 cells were plated ( $1 \times 10^5$  cells per well of 12-well plate) and transfected with 1  $\mu$ g of pLucTKS3 reporter plasmid containing specific responsive elements for Stat3 [20], varied amount of Stat3 siRNA expression vector or negative control vector. For control, DU145 cells were cotransfected with 1  $\mu$ g of Stat3 unresponsive pLucTK reporter plasmid [20] and Stat3 siRNA expression vector or negative control vector. Total amount of DNA transfected was adjusted using empty vector DNA. After 40 hr of incubation, cell extracts were prepared and luciferase activity was determined according to manufacturer's protocols (Promega, Madison, WI). Luciferase activity was normalized per microgram of protein as determined by Bradford assay (Coomassie Plus, Pierce).

### Electromobility Shift Assay

After transfection with either Stat3 siRNA expression vector or negative control plasmid, nuclear extracts were prepared and electromobility shift assay (EMSA) was performed as described previously [10]. For determination of the Stat3 DNA binding activity, nuclear extracts (10  $\mu$ g) were incubated in a final volume of 20  $\mu$ l (10 mM HEPES, pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100  $\mu$ g/ml poly(dI-dC) with radio labeled double stranded Stat3 consensus binding motif 5'-GATCCTCTGGAA-TTCCTAGATC (Santa Cruz Biotechnologies, CA). The protein-DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel at room temperature, and the results were autoradiographed using Molecular Imager FX System (Bio Rad, Hercules, CA).

### Western Blot Analysis

Cell extracts were prepared and resolved on a 10% SDS-PAGE and blotted onto a membrane. After blocking overnight at 4°C in 5% milk in PBS containing 0.1% Tween 20, membranes were incubated overnight

with either antibodies of cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA), Bcl-x<sub>L</sub> (Santa Cruz, CA), or antibodies against Stat3 or phospho-Stat3 proteins (Cell Signaling Technology). Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

### Northern Blot Analysis

Twenty micrograms of total RNAs were electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). PSA cDNA and Stat3 cDNA were labeled with [ $\alpha$ -<sup>32</sup>P] dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) using the Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech). Hybridization was carried out during 3 hr at 65°C in Rapid-hyb buffer (Amersham Pharmacia Biotech). Membranes were washed for 15 min at 65°C in 2  $\times$  SSC, 0.1% SDS (twice), 0.5  $\times$  SSC, 0.1% SDS, and 0.1  $\times$  SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio Rad).

### PSA Protein Analysis

PSA secretion was quantitated by ELISA with the use of anti-PSA as primary antibody as described by the manufacturer's protocol (Beckman Coulter, Fullerton, CA). Equal numbers of cells were plated in phenol red-free RPMI containing 10% FBS. Cells were allowed to attach for 24 hr, then the cells were transfected with either 1  $\mu$ g of Stat3 siRNA or 1  $\mu$ g of control siRNA. After 3 days, 50  $\mu$ l of supernatant was assayed for PSA.

### Statistical Analysis

Values were expressed as the mean  $\pm$  SE. Statistical analyses were performed by one-way ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons, with a  $P < 0.05$  significant.

## RESULTS

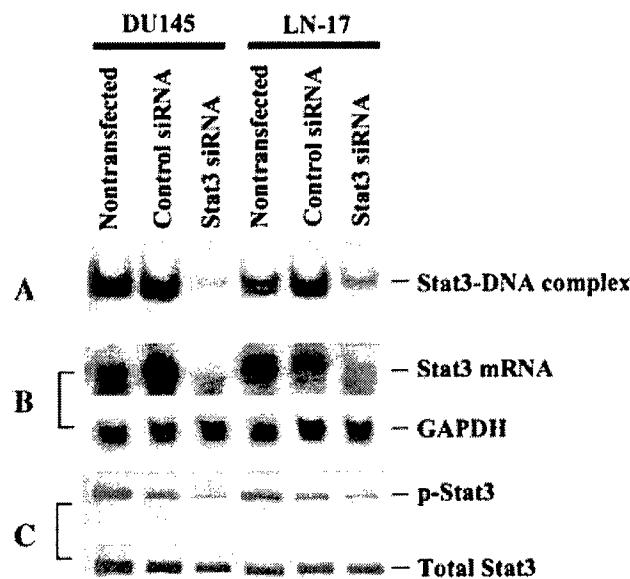
### Stat3 siRNA Inhibits Stat3 DNA-Binding Activity

To determine whether transfection of Stat3 hairpin siRNA vector inhibits Stat3 activity, we used several human prostate cancer cell lines expressing high levels of constitutively activated Stat3 including DU145 and LN-17 cells [8,10]. The LN-17 cells were derived from LNCaP cells that were ectopically transfected with IL-6 cDNA and expressed constitutively activated Stat3 [8,19]. Both cell lines were transfected with either the Stat3 hairpin siRNA vector or the negative control siRNA vector, respectively. Antibiotic G418 (300  $\mu$ g/ml) was added following 24 hr transfection and the cells

were allowed to grow for another 48 hr. The cells were harvested and the Stat3 activity was evaluated by EMSA. The Stat3 hairpin siRNA vector transfected cells demonstrated a marked decrease in formation of Stat3 DNA-protein complex in the gel shift assay compared to the negative control siRNA vector transfected cells (Fig. 1A).

We also examined the effect of Stat3 siRNA on the levels of endogenous Stat3 mRNA. Figure 1B shows that Stat3 siRNA significantly inhibits the steady-state expression levels of Stat3 mRNA compared to that of negative control siRNA vector.

To determine whether the Stat3 siRNA affect the levels of steady-state Stat3 or phosphorylated Stat3 protein, we performed Western blot analysis using antibodies against either Stat3 or phospho-specific Stat3 protein on the cells transfected with either Stat3 siRNA expression vector or negative control siRNA vector. Figure 1C shows that both the levels of steady-state Stat3 and tyrosine-phosphorylated Stat3 protein were decreased by Stat3 siRNA compared to the negative control siRNA vector. It appears that the reduction in steady state levels of total and phosphory-

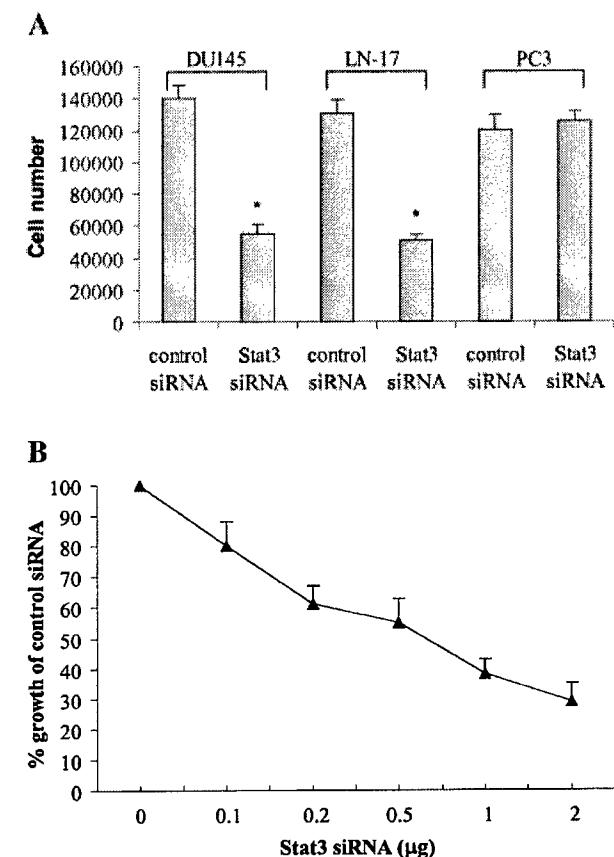


**Fig. 1.** Effect of Stat3 siRNA on Stat3 expression and activity of human prostate cancer cells. **A:** Stat3-DNA binding activity. The Stat3 activity was analyzed using EMSA as described in Materials and Methods. **B:** Stat3 mRNA levels of human prostate cancer cells transfected with Stat3 siRNA or control siRNA were determined by Northern blot analysis. GAPDH expression was used as an internal control. **C:** Effect of Stat3 siRNA on Stat3 protein expression. Western blots were performed using either antibodies against phospho-specific Stat3 (Tyr-705) or total Stat3 with whole cell extracts isolated from prostate cancer cells transfected with Stat3 siRNA expression vector or control siRNA vector.

lated Stat3 is less than the Stat3-DNA binding activity and Stat3 mRNA levels by siRNA Stat3.

### Stat3 siRNA Inhibits Cell Growth and Induces Apoptosis

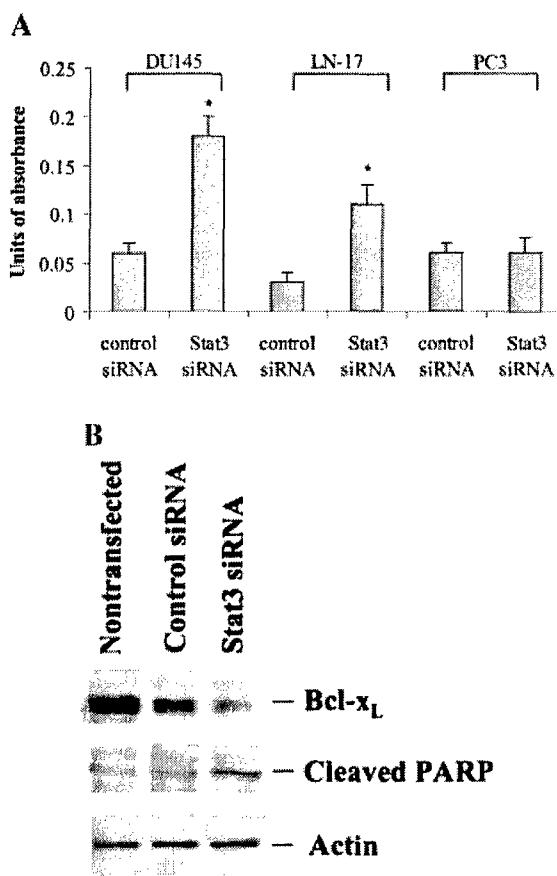
We have previously demonstrated that constitutively activation of Stat3 promotes human prostate cancer cell growth in vitro and tumor growth in vivo [9]. To determine the effect of the Stat3 siRNA on human prostate cancer cell growth, DU145 and LN-17 cells were transiently transfected with either Stat3 siRNA expression vector or the negative control vector. The cells were counted after 4 days of transfection. Figure 2A shows that Stat3 siRNA expression vector inhibited cellular proliferation by 60% in DU145 cells and 50% in LN-17 cells compared to that of the negative



**Fig. 2.** Effect of Stat3 siRNA on human prostate cancer cell growth. **A:** Human prostate cancer DU145, PC3, and LN-17 cells ( $1 \times 10^5$  per well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 24 hr, the cells were transfected with either Stat3 siRNA expression vector or negative control plasmid. Four days later, the cells were counted with a Coulter counter. Columns represent means of data ( $n = 4$ ); bars,  $\pm$ SE. \*, Significantly different from control siRNA. **B:** Transfected with 0.1–2 µg Stat3 siRNA expression vector demonstrated a dose-dependent inhibition of proliferation of DU145 cells. The data are expressed as % of the control siRNA.

control vector. To determine whether the growth inhibition by Stat3 siRNA was dose-dependent, prostate cancer cells were transfected with a range of Stat3 siRNA expression vector (0.1–2 µg/well) and the cell number was determined afterwards. As shown in Figure 2B, the growth inhibition by the Stat3 siRNA was dose-dependent.

Activation of Stat3 protects cells from apoptotic cell death, and blockade of Stat3 activation induces apoptosis [3,6]. Therefore, we evaluated the effect of the Stat3 siRNA on apoptosis in human prostate cancer cells. The Stat3 siRNA induced apoptosis was examined using an apoptosis specific ELISA kit. Figure 3A



**Fig. 3.** Effect of Stat3 siRNA on prostate cancer cell apoptosis. **A:** Cell death analysis by a specific ELISA kit. Human prostate cancer DU145, LN-17, and PC3 cells were transfected with Stat3 siRNA expression vector or control siRNA vector. Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the amount of protein. Data are expressed as mean  $\pm$  SD of four independent experiments. \*, Significantly different from control siRNA. **B:** Western blot analysis of the expression of Bcl-x<sub>L</sub> and cleaved PARP proteins in LN-17 prostate cancer cells transfected with Stat3 siRNA expression vector or control siRNA vector. Immunoblots were prepared from 40 µg of whole cell lysate from LN-17 cells transfected with Stat3 siRNA expression vector or control siRNA vector.

shows that the Stat3 siRNA induced apoptosis in DU145 by threefold and in LN-17 by fourfold compared to the negative control vector. Immunoblots using antibodies against Bcl-x<sub>L</sub> and cleaved PARP proteins were also performed to determine the effect of the Stat3 siRNA on apoptosis. As shown in Figure 3B, the Stat3 siRNA significantly reduced the expression of Bcl-x<sub>L</sub> and enhanced the expression of cleaved PARP compared to the negative control vector, further demonstrating that the Stat3 siRNA induces human prostate cancer cells to undergo apoptotic cell death.

We also evaluated the selectivity of the Stat3 siRNA action. We transfected Stat3 siRNA expression vector into Stat3-negative PC3 human prostate cancer cells. The Stat3 siRNA shows no effect on the proliferation (Fig. 2A) nor apoptosis (Fig. 3A) in PC3 cells compared to the negative control vector, suggesting that the Stat3 siRNA inhibits cell proliferation only in cells that express constitutively activated Stat3.

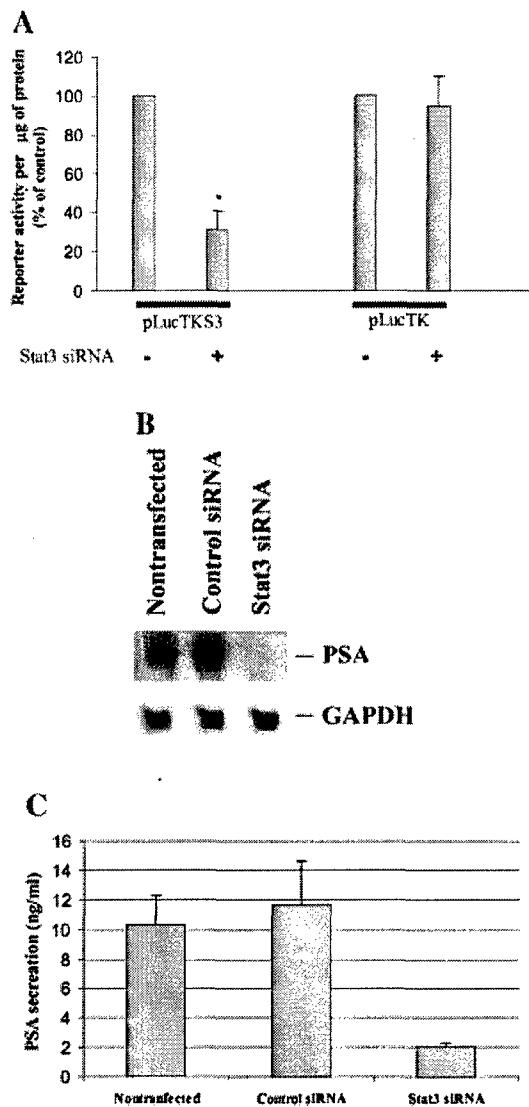
#### Stat3 siRNA Inhibits Stat3-Mediated Gene Expression

Stat3 activation contributes to oncogenesis through regulation of its target genes. To examine the effect of the Stat3 siRNA on Stat3-mediated gene expression, DU145 cells were cotransfected with the pLucTKS3 reporter containing the Stat3 responsive elements [20] and with either Stat3 siRNA expression vector or negative control vector, and cytosolic extracts were prepared for luciferase assays. The pLucTK reporter that contains no Stat3 responsive element was used as a control [20]. As shown in Figure 4A, the Stat3 siRNA significantly inhibited the induction of the Stat3-dependent pLucTKS3 luciferase reporter activity without affecting the Stat3-independent pLucTK activity compared to the negative control vector.

We previously demonstrated that activated Stat3 enhances the expression of AR-mediated genes including PSA [9]. To test the effect of the Stat3 siRNA on PSA expression, Northern blot analyses were performed to compare the levels of the expression of PSA mRNA in LN-17 cells that have been transfected with either Stat3 siRNA expression vector or negative control vector. As shown in Figure 4B,C, the Stat3 siRNA significantly reduced the levels of PSA expression compared to the negative vector control.

#### DISCUSSION

Studies to date provide strong evidence that aberrant Stat3 signaling may play an important role in the development and progression of prostate cancer. We previously demonstrated increase Stat3 activation in prostate cancer and that constitutively activated Stat3 promotes prostate cancer cell tumor growth [5,9].



**Fig. 4.** Effect of Stat3 siRNA on Stat3-mediated gene expression. **A:** DU145 cells expressing constitutively activated Stat3 were cotransfected with the pLucTKS3 reporter (1  $\mu$ g) containing the Stat3 responsive elements and with either 1  $\mu$ g of Stat3 siRNA expression vector (+) or 1  $\mu$ g of negative control vector (-). Cell extracts were prepared for luciferase assays. The pLucTK reporter that contains no Stat3 responsive element was used as a control. Luciferase activity was determined according to manufacturer's protocols and normalized per  $\mu$ g of protein. Data are representative of three independent experiments. \*, Significantly different compared to control siRNA. **B:** Stat3 siRNA inhibits PSA mRNA expression in LN-17 cells. Total RNA was isolated from LN-17 cells transfected with either Stat3 siRNA expression vector or control siRNA vector and used for Northern blot analysis as described in Materials and Methods. GAPDH was used as a loading control. **C:** Stat3 siRNA inhibits PSA protein expression in LN-17 cells. The levels of PSA protein expression in the medium was analyzed by PSA ELISA from LN-17 cells transfected with either Stat3 siRNA expression vector or control siRNA vector.

Blockade Stat3 activation by a dominant negative Stat3 mutant resulted in suppression of prostate cancer growth both in vitro and in vivo [10]. Numerous studies also demonstrate that Stat3 activates AR-mediated gene expression and prevents cell from apoptosis [6,7,9]. Collectively, these findings indicate that targeting Stat3 signaling may represent a novel approach to treat prostate cancer.

RNAi represents a promising new technology that could have therapeutic applications for the treatment of diseases including cancer by blocking the action of transcription factors and oncogenes with selective silencing of gene expression with exquisite precision and high efficacy [21]. In this study, we have identified the Stat3 siRNA that specifically inhibits constitutively activated Stat3 and suppresses cell proliferation and induces apoptosis. To compare the relative potency of this siRNA Stat3 with Jak/Stat3 inhibitor AG490, LN-17 cells were transfected with 1  $\mu$ g of Stat3 siRNA expression plasmid and the cell number was determined, while the same cells were treated with different doses of AG490 (range from 0 to 50  $\mu$ M). The effect of inhibition of cell growth by expression of 1  $\mu$ g of Stat3 siRNA (about 60% inhibition) is similar to the effect of 30  $\mu$ M of AG490.

Previous studies demonstrated that Stat3 is constitutively activated in human prostate cancer compared to normal prostate [5,6], and that constitutively activated Stat3 promotes prostate cancer cell growth both in vitro and in vivo [9]. This study showed that the Stat3 siRNA only inhibits the proliferation and induces apoptosis in cells expressing constitutively activated Stat3, but not in Stat3-inactive PC3 cells, further demonstrating the selectivity of the Stat3 siRNA and potential therapeutic utility of the Stat3 siRNA for prostate cancer expressing active Stat3.

The PSA is synthesized primarily by normal and malignant prostate and the levels of PSA in serum correlate with the clinical stage of the disease. We have previously demonstrated that constitutively activated Stat3 enhances PSA expression in vitro and in vivo and enhances PSA transcription [9]. We showed here that blockade of Stat3 activation by the Stat3 siRNA significantly inhibited PSA mRNA expression in LN-17 cells (Fig. 4B), indicating that targeting Stat3 activation by the Stat3 siRNA could inhibit the AR-mediated gene expression in prostate cancer cells.

In conclusion, we have identified the Stat3 siRNA that specifically inhibits constitutively activated Stat3 and suppresses cell proliferation and induces apoptosis. The Stat3 siRNA resulted in blockade of Stat3 DNA-binding activity and inhibited the levels of Stat3 mRNA. Targeting Stat3 activation with RNAi holds therapeutic promise for prostate cancer with constitutive Stat3 activation. Several strategies have

been reported to block Stat3 activation including using Stat3 decoy oligonucleotides in head and neck cancer cells [13]. Although siRNA method has emerged as powerful RNAi reagents for directed posttranscriptional gene silencing and treatment of viral diseases and cancer, it would be of interest to compare the relative therapeutic potency of using Stat3 decoy oligonucleotides with the Stat3 siRNA identified in this study in prostate cancer cells.

### ACKNOWLEDGMENTS

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# Interleukin-6 Protects LNCaP Cells From Apoptosis Induced by Androgen Deprivation Through the Stat3 Pathway

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**BACKGROUND.** Elevated expression of interleukin-6 (IL-6) is implicated in the progression of hormone refractory prostate cancer. Previous studies demonstrated that IL-6 promotes androgen-independent growth of prostate cancer cells. In this study, the effect of IL-6 on apoptosis induced by androgen deprivation was investigated.

**METHODS.** The effect of IL-6 on apoptosis induced by androgen deprivation in LNCaP cells was examined by cell death ELISA and Western blot using cleaved poly (ADP-ribose) polymerase (PARP) and caspase-9, as well as Bcl-x<sub>L</sub> and phosphorylated Bad. The Stat3 in IL-6-mediated anti-apoptosis in prostate cancer cells was examined using either dominant-negative or constitutively activated Stat3 mutants.

**RESULTS.** Overexpression of IL-6 renders androgen sensitive LNCaP human prostate cancer cells more resistant to apoptosis induced by androgen deprivation. LNCaP cells undergo apoptosis after 72 hr of androgen deprivation, an outcome is largely absent in clones overexpressing IL-6 as measured by cell death ELISA and chromatin degradation assays. IL-6 over-expressing cells resulted in a significant decrease in the expression of cleaved PARP and cleaved caspase-9 as well as an increase in the expression of Bcl-x<sub>L</sub> and phosphorylated Bad. Addition of IL-6 antibody completely abolished the anti-apoptotic activity of IL-6. This protective effect of IL-6 was reversed by the expression of a dominant-negative Stat3 mutant, Stat3F. Furthermore, ectopic expression of a constitutively active Stat3 antagonized androgen deprivation-induced cell death of LNCaP cells.

**CONCLUSION.** These results indicate that IL-6 protects androgen sensitive LNCaP cells from apoptosis induced by androgen deprivation, and Stat3 activation play an important role in IL-6-mediated anti-apoptosis in prostate cancer cells. *Prostate* 60: 178–186, 2004.

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**KEY WORDS:** IL-6; prostate cancer; apoptosis; Stat3; androgen-independence

## INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and second leading cause of cancer death in American men. Initially, the growth of prostate epithelial cells is dependent on androgen. When androgen is depleted, these cells undergo apoptosis and die, the basis for androgen ablation therapy, a common treatment for prostate cancer [1,2]. However, most patients will relapse to hormone refractory disease due to the growth of androgen-independent cancer cells. In this stage, cells are more resistant to apoptotic cell death and thus androgen ablation is ineffective [2].

Interleukin-6 (IL-6) is a 21–28 Kd multifunctional cytokine involved in many cellular processes such as

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inflammation, cell differentiation, and proliferation [3]. IL-6 production correlates with tumor progression in human cancer such as pleural mesothelioma, glioblastoma, and ovarian and prostate cancer [4–7]. The expression of IL-6 and its receptor is consistently demonstrated in human prostate cancer cell lines and in freshly isolated human prostate carcinoma and benign prostate hyperplasia [8–10]. Clinically, the levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer [4,11]. IL-6 has been suggested to have both growth-promoting and inhibiting activities in androgen-sensitive LNCaP human prostate cancer cells *in vitro* [12–15]. IL-6 activates AR-mediated gene expression in LNCaP cells *in vitro* [14,16–18], suggesting that IL-6 may play a critical role during the progression of prostate cancer. In addition, overexpression of IL-6 in androgen sensitive LNCaP human prostate cancer cells promotes LNCaP cell androgen-independent growth *in vitro* and *in vivo* [19].

The biological activities of IL-6 are mediated by the IL-6 receptor. IL-6 receptor is composed of two components, a 80 Kd transmembrane protein that has ligand-binding capacity and a 130 Kd membrane glycoprotein (gp130) that mediates signal transduction following IL-6 binding [20]. The IL-6 receptor is abundant in many types of prostate cells including both androgen-dependent and androgen-independent cells [10]. In addition to the transmembrane IL-6R, a soluble IL-6 form of IL-6R (sIL-6R) can be generated either from truncated membrane protein or translation from an alternatively spliced mRNA [21]. IL-6 can bind to this soluble form of the receptor, an alternate target for the biologic activity [21]. IL-6 signaling through gp130 transduces signals into the interior of the cell through several major signaling pathways including the Janus kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathway [22], mitogen-activated protein (MAP) kinase pathways [23], and the phosphatidylinositol (PI) 3-kinase-AKT pathways [24].

Apoptosis is a physiological cellular suicide program that maintains tissue homeostasis with pro-apoptotic and anti-apoptotic protein family members implicated in cell survival/death decisions. The role of IL-6 in the regulation of apoptosis is demonstrated in many cancer cells. Overexpression of IL-6 increases anti-apoptotic activity and thereby tumorigenic potency in basal cell carcinoma [25]. IL-6 can regulate the anti-apoptotic Bcl-2 family proteins, and the expression of Mcl-1, a Bcl-2 family member, was significantly induced by IL-6 [26].

While IL-6 plays a critical role in the development of androgen independent prostate cancer, the molecular mechanisms of IL-6 mediated androgen independence are largely unknown. In this study, we demonstrated

that IL-6 can protect androgen-sensitive LNCaP human prostate cancer cells from apoptotic death induced by androgen depletion. Furthermore, the anti-apoptotic activity of IL-6 is mediated by Stat3 signaling pathway.

## MATERIALS AND METHODS

### Tissue Culture

The LNCaP cells were maintained in RPMI 1640 supplemented with 10% of FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37°C in 5% CO<sub>2</sub> incubator. Transfection and selection of LNCaP cells stably expressing IL-6 were described previously [13]. The clones overexpressing IL-6 (LN-S15, LN-S17) and its neo control (transfected with vector alone) cells were cultured in the same medium but containing 0.3 mg/ml of G418. In order to investigate the effect of androgen deprivation, cells were cultured in a medium containing 10% charcoal-stripped FBS (CS-FBS) instead of regular 10% FBS.

### In Vitro Growth Assay

LNCaP and IL-6 over-expressing cells (LN-S15 and LN-S17) (10<sup>4</sup>/well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 2 days in regular culture medium with 10% FBS, cells were switched into RPMI 1640 medium containing either 10% FBS or 10% CS-FBS (Hyclone, UT). Three days later, cells were trypsinized and counted with a Coulter counter. For the IL-6 antibody test, 20 µg of IL-6 antibody (Sigma Chemicals Co.) was added per ml of culture medium.

### Apoptosis ELISA Test

The ELISA kit was obtained from Roche Molecular Biochemicals (Indianapolis, IN) and the protocol was followed according to the manufacturer's instructions. For the preparation of samples, LNCaP and IL-6 over-expressing cells (LN-S15 and LN-S17) (10<sup>4</sup>/well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 2 days in regular culture medium with 10% FBS, cells were switched into a medium containing either 10% FBS or 10% CS-FBS (Hyclone). Three days later, cell lysates were obtained and cell death was analyzed and the absorbance values were normalized by the cell number.

### Fluorescent Microscopic Studies

Cells (5 × 10<sup>3</sup>) were plated in microslides in normal medium for 2 days and switched into a medium containing CS-FBS after washing. Three days later, cells were fixed in 8% paraformaldehyde for 15 min, washed and stained with Hoechst 33258 solution (5 µg/ml in PBS, 5 min). Cells were then washed with PBS, mounted and examined under fluorescent microscope.

### Transfection

LNCaP, LN-S15, and LN-S17 cells were transiently transfected with 2  $\mu$ g of either a dominant negative Stat3 (Stat3F) or a constitutively activated Stat3 mutant (Stat3c) using SuperFect Transfection Reagent (QIAGEN, Inc., Valencia, CA). Briefly, cells ( $1 \times 10^6$ ) were plated into 6-well plates and allowed to adhere for 24 hr. Cells were transfected with either Stat3F or Stat3c in serum-free medium for 4 hr, incubated with complete medium for 24 hr, and then were switched into a medium containing CS-FBS. Three days later, the cell lysates were prepared and used for the quantitation of apoptosis by the ELISA kit, growth assay, Western blot analysis, and electromobility shift assay (EMSA). For controls, the same amount of empty vector was used for transfection.

### EMSA

Whole cell extracts were prepared and EMSA was performed as described previously [27]. For determination of the Stat3 DNA binding activity, whole cell extracts (20  $\mu$ g) were incubated in a final volume of 20  $\mu$ l (10 mM HEPES, pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100  $\mu$ g/ml poly(dIdC) with radiolabeled double stranded Stat3 consensus binding motif 5'-GATCCTCTGGGAATTCTAGATC (Santa Cruz Biotechnologies, CA). The protein-DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel at room temperature, and the results were autoradiographed using Molecular Imager FX System (Bio Rad, Hercules, CA).

### Western Blot

Whole cell extracts were obtained, as described previously [19], and resolved in 8–12% SDS-PAGE depending on the molecular weight of the protein to be detected. After blocking overnight 4°C in 5% milk in PBS-0.1% Tween 20, membranes were incubated overnight with antibodies of cleaved caspase-9 (Cell Signaling Technology, MA), cleaved poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology), Bcl-x<sub>L</sub> (Santa Cruz Biotechnologies), or phosphorylated Bad (Cell Signaling Technology). Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

### Statistical Analysis

Values were expressed as the mean  $\pm$  SE. Statistical analyses were performed by one-way ANOVA, followed by the Student-Newman-Keuls test for multiple comparisons, with a  $P < 0.05$  significant.

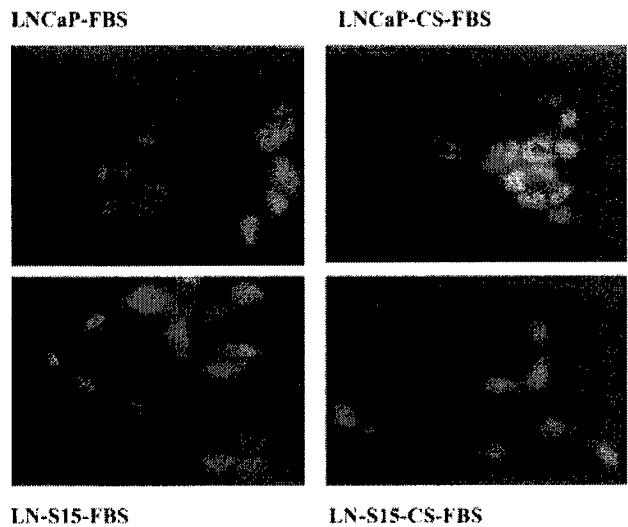
## RESULTS

### Overexpression of IL-6 in LNCaP Cells Confers Resistance to Androgen Deprivation-Induced Apoptosis

Previous studies have demonstrated that the androgen sensitive LNCaP cells express the IL-6 receptor, but express no detectable IL-6 protein [12–15,28]. To test the effect of IL-6 on LNCaP cell function, we ectopically expressed IL-6 by introduction of a full-length IL-6 cDNA into IL-6-negative LNCaP cells as described previously [13]. Two stable IL-6 independent transfectants (LN-S15 and LN-S17) were isolated that express high levels of IL-6 (2,465 and 2,743 pg/ml/10<sup>6</sup> cells, respectively) with a vector-alone control (neo). We previously demonstrated that the growth of androgen sensitive LNCaP cells in culture was reduced by about 50% after 48 hr in androgen-deprived charcoal-stripped serum condition compared with that in the normal serum condition [19]. However, in the IL-6 overexpression cells, there was only a 5–10% decrease in growth under these androgen-deprived conditions compared with growth in normal serum, suggesting that overexpression of IL-6 can enhance the growth of LNCaP cells in the androgen deprived condition *in vitro*.

Androgen deprivation triggers apoptosis in both normal and malignant androgen-dependent prostate epithelial cells. LNCaP cells are androgen sensitive human prostate cancer cells. To test whether LNCaP cells undergo programmed cell death in androgen deprived condition *in vitro*, and whether IL-6 can prevent apoptosis, LNCaP cells, LNCaP-neo, and IL-6 overexpression cells (LN-S15 and LN-S17) were cultured in RPMI 1640 with 10% FBS and then switched to RPMI 1640 with 10% CS-FBS in which physiological levels of androgen were deprived. Death was assessed 72 hr later through different techniques. The characteristic morphology of apoptosis was determined by staining with Hoechst 33258. Figure 1 shows apoptotic morphologies of the representative parental LNCaP and IL-6 overexpressing LN-S17 cells in androgen deprived conditions. A typical apoptotic morphology including chromatin condensation and nuclear fragmentation was clearly observed in the parental LNCaP and neo control cells cultured in the CS-FBS conditions, but not in IL-6-over-expressing cells (LN-S15 and LN-S17).

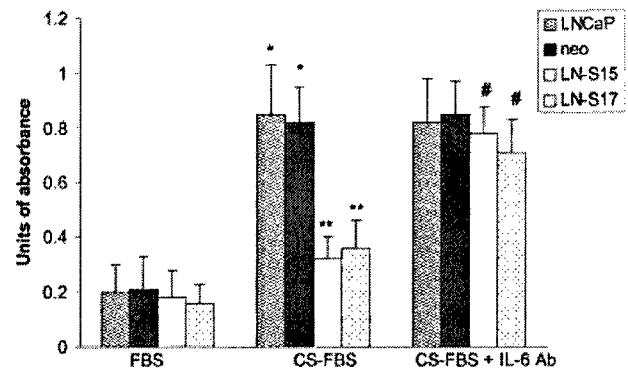
Apoptotic cell death was determined using the apoptosis specific ELISA assay to evaluate DNA fragmentation. As shown in Figure 2, parental LNCaP and neo control cells showed significant levels of apoptotic death in CS-FBS as compared to normal FBS ( $P < 0.01$ ), whereas IL-6 over-expressing cells showed a significant lower level of apoptotic death as compared to parental LNCaP and neo control cells ( $P < 0.01$ ).



**Fig. 1.** Expression of interleukin-6 (IL-6) protects LNCaP cells against apoptosis induced by androgen deprivation. Parental LNCaP and IL-6 overexpressing LN-S15 cells (LN-S15) were cultured in either normal FBS or androgen deprived CS-FBS conditions for 3 days. The cells were stained with Hoechst 33258 fluorescent dye and examined by fluorescent microscope. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

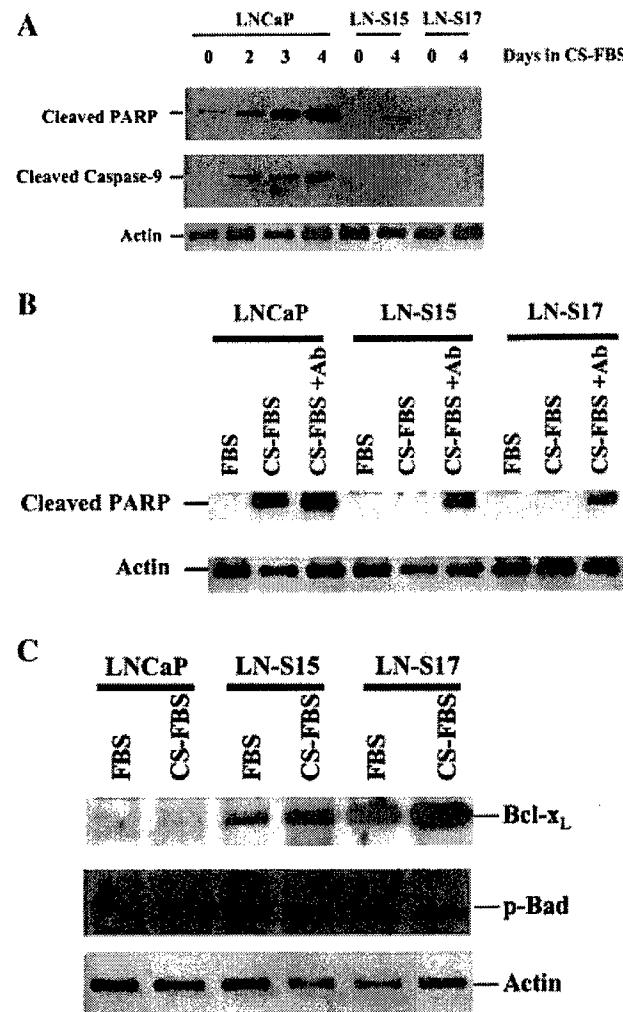
Addition of IL-6 antibody to the cell culture media almost completely blocked the anti-apoptotic activity of IL-6 ( $P < 0.01$ ), indicating that the anti-apoptotic effect was mediated specifically by IL-6.

Immunoblots using antibodies against several pro-apoptotic and anti-apoptotic proteins were performed to determine the effect of IL-6 on apoptosis. When apoptosis occurs, caspases are cleaved into active



**Fig. 2.** Cell death analysis by a specific ELISA kit. Parental LNCaP, neo, and IL-6 overexpressing (LN-S15 and LN-S17) cells were cultured in either normal FBS, androgen deprived CS-FBS, or CS-FBS plus 20  $\mu$ g/ml of IL-6 antibody conditions. Data are expressed as mean  $\pm$  SE of four independent experiments. \*,  $P < 0.01$  compared with neo control cells cultured in normal FBS conditions; \*\*,  $P < 0.01$  compared with neo controls in CS-FBS conditions; #,  $P < 0.01$  compared with LN-S15 and LN-S17 in the CS-FBS condition in the absence of the IL-6 antibody.

enzymes from inactive precursors and PARP is also cleaved. When parental LNCaP cells or neo control LNCaP cells were androgen deprived, elevated levels of the cleaved PARP and cleaved caspase-9 were detected (Fig. 3A). However, in IL-6 overexpressing LN-S15 and LN-S17 cells significantly lower levels of cleaved PARP and cleaved caspase-9 were observed as compared to the parental LNCaP cells or to the neo control cells when grown in androgen deficient



**Fig. 3.** Effect of IL-6 on the expression of pro-apoptotic and anti-apoptotic proteins in LNCaP cells in androgen deprivation conditions. Immunoblots were prepared from 40  $\mu$ g of whole cell lysate from parental LNCaP, neo, and IL-6 overexpressing LNCaP clone LN-S15 and LN-S17 cells cultured either in normal FBS or androgen deprived charcoal-stripped FBS (CS-FBS) conditions for 3 days as indicated. **A:** Immunoblots were analyzed with cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-9 antibodies. **B:** Twenty microgram per milliliter of IL-6 antibody were added to the androgen deprived charcoal stripped culture medium (CS-FBS-Ab), and whole cell lysate were immunoblotted with cleaved PARP antibody. **C:** Immunoblots were analyzed with Bcl-x<sub>L</sub> and phospho-Bad antibodies.

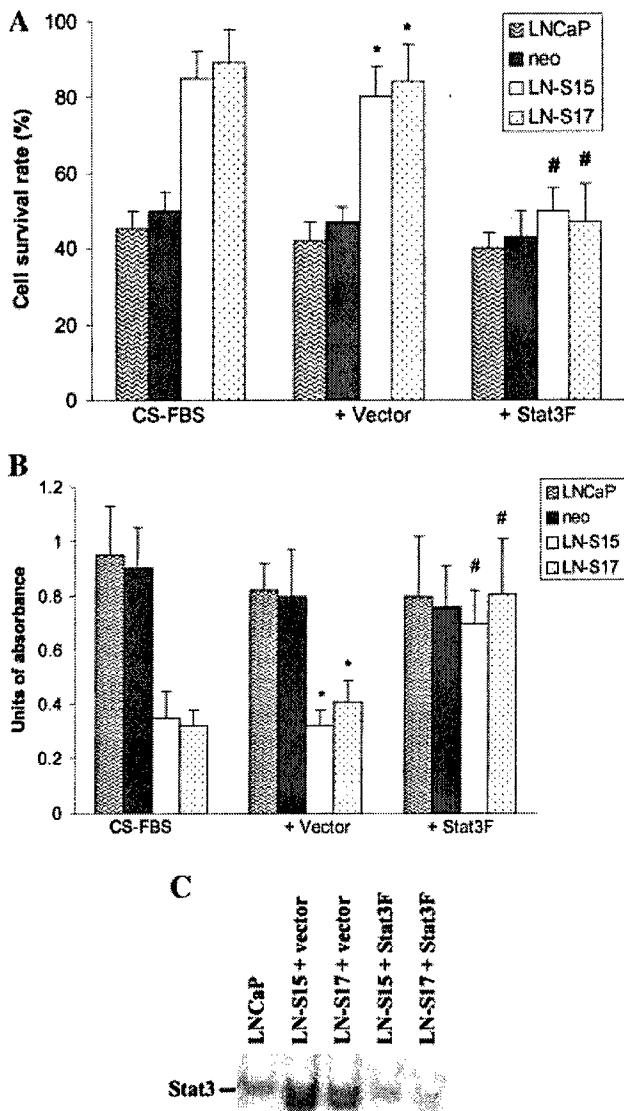
conditions (Fig. 3A). Addition of IL-6 antibody to the IL-6 overexpressing LN-S15 and LN-S17 cell culture media significantly elevated the levels of cleaved PARP expression (Fig. 3B), further suggesting a role for IL-6 specifically. Concomitantly, the increase in anti-apoptotic protein was observed in LN-S15 and LN-S17 cells. When cells were grown in androgen-deficient media, the expression of Bcl-x<sub>L</sub> and phosphorylated Bad proteins was significantly increased in the IL-6 overexpressing LN-S15 and LN-S17 cells compared to parental LNCaP cells (Fig. 3C). Collectively, these data demonstrate that LNCaP cells undergo apoptotic cell death upon androgen withdrawal, and that IL-6 protects these cells from androgen deprivation induced apoptosis.

#### Stat3 Activation Mediates Anti-Apoptotic Activity of IL-6

We have previously demonstrated that IL-6 significantly activates Stat3 in LNCaP cells both with androgen (normal FBS) and without androgen (CS-FBS) [19]. To elucidate the mechanism underlying this anti-apoptotic effect of IL-6, we investigated whether activation of Stat3 by IL-6 is responsible for this effect. We transfected a dominant-negative Stat3 mutant, Stat3F, into the LNCaP parental, neo, and IL-6 overexpressing cells. The dominant-negative Stat3 construct carries a phenylalanine substitution of the tyrosine residue at 705 that causes a reduction of the tyrosine phosphorylation of wild type Stat3 and inhibits the action of endogenous Stat3 [27,29,30]. As

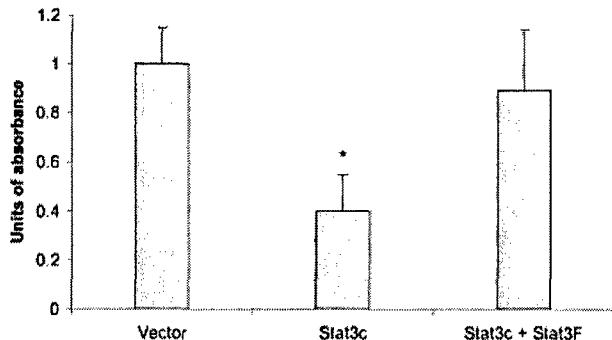
**Fig. 4.** Stat3F inhibits anti-apoptotic activity of IL-6. **A:** Stat3F inhibits IL-6 induced LNCaP cell survival in androgen deprived media. Parental LNCaP, neo, IL-6 overexpressing LN-S15 and LN-S17 cells were cultured in RPMI 1640 supplemented with 10% CS-FBS. The cells were then transiently transfected with equal amounts (2  $\mu$ g) of either vector controls or Stat3F. The cell numbers were counted after 3 days in CS-FBS condition and cell survival values were expressed as % relative to the complete FBS. **B:** Stat3F blocks anti-apoptotic activity of IL-6 in androgen deprived conditions. Parental LNCaP, neo, IL-6 overexpressing LN-S15 and LN-S17 cells were cultured in RPMI 1640 supplemented with 10% CS-FBS. The cells were then transiently transfected with equal amounts (2  $\mu$ g) of either vector controls or Stat3F. Quantitation of apoptosis by a special ELISA kit in these cells after cultured in androgen deprived CS-FBS for 3 days. Data are expressed as mean  $\pm$  SE of four independent experiments. \*,  $P < 0.05$  compared with neo control in CS-FBS conditions; #,  $P < 0.05$  compared with LN-S15 and LN-S17 transfected with vector control in the CS-FBS condition. **C:** Effect of Stat3F on Stat3 activity. IL-6 overexpressing LN-S15 and LN-S17 cells were transfected with either vector alone or Stat3F. After transfection, the cells were continuously cultured in androgen deprived condition for 3 days and the cell extracts were prepared. The Stat3 activity was analyzed using electromobility shift assay (EMSA) as described in "Materials and Methods."

seen in Figure 4A, when Stat3F was transfected, the anti-apoptotic activity of IL-6 was abolished in IL-6 overexpressing LN-S15 and LN-S17 cells. When these cells were put into media without androgen, the survival rates were greater than 85%. In cells transfected with Stat3F, survival rates were less than 50%, a level observed in the parental LNCaP and neo control cells grown in the absence of androgen. The effect of transfecting expression of Stat3F on apoptosis was also examined by ELISA. As shown in Figure 4B, transient transfection of Stat3F into IL-6 overexpressing LN-S15 and LN-S17 cells resulted in a significant increase in apoptotic death ( $P < 0.01$ ). Figure 4C demonstrated that the levels of Stat3 activity in these IL-6 overexpressing LN-S15 and LN-S17 clones were significantly decreased after the transient transfection of Stat3F. These studies confirm that blocking Stat3 activation reverses the anti-apoptotic activity of IL-6.



### Stat3 Activity Antagonizes Androgen-Deprivation Induced Death in LNCaP Cells

The anti-apoptotic activity of IL-6 from androgen deprivation appears to be mediated by Stat3 activation in LNCaP cells. We further investigated whether increased Stat3 activity might be sufficient to protect cells from androgen deprivation-induced apoptosis. To demonstrate a direct effect of enhanced Stat3 activity on LNCaP cell apoptosis induced by androgen deprivation, LNCaP cells were transfected with a constitutively activated Stat3 mutant, Stat3c, and vector control, respectively. Stat3c is a constitutively activated Stat3 (a mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3) that induces cellular transformation and tumor formation in nude mice [31]. Previous studies have demonstrated that ectopic expression of Stat3c into LNCaP cells enhanced Stat3 activation and promoted cell androgen independent growth [32]. After transfection with Stat3c, the cells were deprived of androgen for 72 hr, starting 12 hr after transfection, and the cell lysis was collected. Cell death was determined by the ELISA assay. As shown in Figure 5, overexpression of constitutively activated Stat3 in LNCaP cells resulted in significantly less cell death as compared to the vector control cells ( $P < 0.01$ ) without androgen, and addition of the dominant-negative Stat3 (Stat3F) restored the level of cell death to that of the vector control, suggesting that the constitutive activation of Stat3 is sufficient to protect LNCaP undergoing apoptosis induced by androgen deprivation.



**Fig. 5.** Constitutively activated Stat3 protects LNCaP cells apoptosis induced by androgen deprivation. LNCaP cells were cultured in RPMI 1640 supplemented with 10% CS-FBS. The cells were then transiently transfected with equal amounts (2  $\mu$ g) of either vector controls or constitutively activated Stat3c or Stat3c plus Stat3F. Quantitation of apoptosis by a special ELISA kit was performed after cultured in androgen deprived CS-FBS for another 3 days. Data are expressed as mean  $\pm$  SE of four independent experiments. \*,  $P < 0.05$  compared with vector control in CS-FBS conditions.

### DISCUSSION

In this study, we have investigated the molecular mechanisms by which IL-6 promotes androgen independent progression of prostate cancer cells. We demonstrated that IL-6 protects androgen sensitive LNCaP cells from androgen deprivation induced apoptosis through activation of Stat3 signaling pathway.

We previously demonstrated that overexpression of IL-6 in androgen sensitive LNCaP cells enhances androgen independent growth in vitro and in vivo [19]. To understand the mechanism of IL-6 induced androgen independent growth of LNCaP cells, we analyzed the effect of IL-6 on apoptosis induced by androgen deprivation. Several lines of evidence from this study demonstrate that overexpression of IL-6 can protect androgen deprivation-induced apoptosis in LNCaP cells. First, overexpression of IL-6 rescued LNCaP cells from cell death induced by androgen withdrawal. Second, LNCaP cells underwent apoptosis 72 hr androgen was removed, an outcome is largely absent in clones overexpressing IL-6. Third, IL-6 over-expressing cells resulted in a significant decrease in the expression of pro-apoptotic proteins such as cleaved PARP and cleaved caspase-9, and an increase in the expression of the anti-apoptotic proteins Bcl-x<sub>L</sub> and phosphorylated Bad compared to the parental LNCaP cells. Forth, addition of IL-6 antibody completely abolished the anti-apoptotic activity of IL-6, suggesting that anti-apoptosis is specifically mediated by IL-6.

IL-6 has been implicated in the modulation of growth and differentiation in many malignant tumors and is associated with poor prognosis in several solid and hematopoietic neoplasms such as renal cell carcinoma, ovarian cancer, lymphoma, and melanoma [33]. The role of IL-6 in prostate cancer development and progression has been a subject of intensive investigation. The expression of IL-6 and its receptor has been consistently demonstrated not only in human prostate cancer cell lines but more importantly in human prostate carcinoma and benign prostate hyperplasia obtained directly from patients [8–10]. Clinically, the levels of IL-6 in the sera are significantly elevated in the patients with hormone refractory and metastatic prostate cancer compared to hormone sensitive prostate cancer [4,11]. Multiple studies have demonstrated that IL-6 is elevated in the sera of patients with metastatic prostate cancer and that the levels of IL-6 correlate with tumor burden as well as serum PSA or clinically evident metastases [4,11]. Collectively, these clinical data suggest that elevated IL-6 levels are associated with prostate cancer progression to an androgen-independent phenotype.

In addition to the clinical data on the role of IL-6 in androgen independent prostate cancer, experimental

studies demonstrate that IL-6 plays a critical role in prostate cancer cell growth and differentiation. IL-6 can function as a paracrine growth factor for the human LNCaP androgen-sensitive prostate cancer cells and an autocrine growth factor for the human DU145 and PC-3 androgen-insensitive prostate cancer cells [12]. IL-6 can activate AR-mediated gene expression by activation of the AR through a Stat3 pathway in LNCaP cells [13,16,17]. Our previous studies demonstrated that overexpression of IL-6 increases PSA mRNA expression and enhances AR activation in LNCaP cells [19]. In addition, overexpression of IL-6 promotes androgen independent growth of androgen sensitive LNCaP cells in vitro and in vivo [19]. These studies demonstrate that IL-6 activates AR signaling in a ligand-independent manner and induces a synergistic AR response with very low concentrations of androgen.

Accumulating evidence has demonstrated that abnormal AR signaling has contributed to prostate cancer androgen independent growth. Several reports suggested that the AR can be activated by growth factors and cytokines to display enhanced activity in the presence of androgen or to function even in the absence of androgen [34–37]. Recently, results from a number of groups demonstrated that IL-6 activates AR-mediated gene expression by activation of the AR through a Stat3 pathway in LNCaP cells [13,14,16,17]. Induction of neuroendocrine differentiation has also been suggested in the androgen independent prostate cancer cells [38,39]. IL-6 can induce LNCaP cell neuroendocrine differentiation mediated by the activation of Stat3 and MAPK signalings [40–42]. Androgen withdrawal triggers apoptosis in both normal and malignant androgen-dependent prostate epithelial cells. However, androgen-refractory prostate cancer cells do not undergo apoptosis [1]. Thus alteration of apoptotic signaling pathways should be critical for the survival of androgen-refractory prostate cancer cells. In this study, we demonstrate that IL-6 protects LNCaP cells from undergoing apoptotic death induced by androgen deprivation.

The effect of IL-6 on cell proliferation, differentiation, and survival is mediated by differential activation of several major signaling pathways including JAK-STAT, MAPK, and PI3K-Akt. The question arises as to what is the mechanism of anti-apoptosis induced by IL-6 in LNCaP cells. Apoptosis can be induced in response to various cytotoxic stimuli including cytokines and growth factors. These stimuli activate a series of tightly controlled intracellular signals including Stat3. The role of Stat3 in the protection of apoptosis has been already suggested in many cancer cells including prostate [43]. In U266 myeloma cells, constitutive activation of Stat3 signaling confers resistance to apoptosis [44]. In addition, Stat5 and Stat3 have also been demonstrated

to activate the Bcl-x<sub>L</sub> expression directly [44,45]. In this study, we have clearly shown that the anti-apoptotic activity of IL-6 is mediated mainly by Stat3 signaling. We demonstrated that IL-6 overexpressing cell clones are protected from androgen-deprivation induced apoptosis through activation of Stat3 signaling. This protective effect of IL-6 was reversed by the expression of a dominant-negative Stat3 mutant, Stat3F. Furthermore, ectopic expression of a constitutively active Stat3 antagonized androgen deprivation-induced cell death of LNCaP cells.

In conclusion, we demonstrate that overexpression of IL-6 protects androgen sensitive LNCaP prostate cancer cells from apoptotic death induced by androgen deprivation through activation of Stat3 signaling pathway. Since both IL-6 levels are significantly elevated in hormone refractory prostate cancer and Stat3 activity is elevated in prostate cancer [46], targeting IL-6/Stat3 signaling may be of therapeutic value in the treatment of androgen independent prostate cancer.

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## Interleukin-6 Promotes Androgen-independent Growth in LNCaP Human Prostate Cancer Cells<sup>1</sup>

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### ABSTRACT

**Purpose:** Prostate cancer frequently progresses from an initial androgen dependence to androgen independence, rendering the only effective androgen ablation therapy useless. The mechanism underlying the androgen-independent progression is incompletely understood. Interleukin (IL)-6 has been implicated in this androgen-independent progression. In this study, we tested whether IL-6 induces androgen-independent growth both *in vitro* and *in vivo*.

**Experimental Design:** IL-6 was expressed in androgen-sensitive LNCaP cells. The effects of IL-6 on androgen receptor activity was determined by Northern blots and gel shift assays. The effects of IL-6 on LNCaP cell growth were determined *in vitro* by MTT assay and *in vivo*.

**Results:** IL-6 can enhance the growth of androgen-sensitive LNCaP cells in the androgen-deprived condition *in vitro*, which is accompanied by elevation of androgen-regulated prostate-specific antigen mRNA expression. IL-6 promotes androgen-sensitive LNCaP cell tumor growth in the castrated male mice. IL-6 enhances androgen receptor DNA binding activity and nuclear translocation. The androgen-independent phenotype induced by IL-6 in LNCaP cells is accompanied by significant activation of signal transducers and activators of transcription 3 and mitogen-activated protein kinase signal pathways.

**Conclusions:** These studies clearly provide experimental evidence that IL-6 initiates and/or enhances the transition of prostate cancer cells from an androgen-dependent to an androgen-independent phenotype.

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### INTRODUCTION

The growth of prostate epithelial cells requires a physiological level of androgen, both to stimulate proliferation and inhibit apoptotic death (1). Androgen binds to the AR,<sup>4</sup> which triggers interaction of AR to specific AREs in the promoters of androgen-regulated genes. These interactions facilitate the activation or repression of genes regulating development, differentiation, and proliferation of prostate epithelial cells. Currently, the standard treatment for metastatic prostate cancer is androgen ablation therapy. The problem is that whereas almost all patients with prostate cancer initially respond to androgen ablation therapy, virtually every patient will relapse to hormone-refractory disease due to the growth of androgen-independent cancer cells, rendering the only effective therapy useless. The molecular cause of acquired androgen-independent growth, which is promoted by activation of AR signaling through AR gene mutation and amplification (2, 3), coactivators (4), and cross-talk between the AR and protein kinase pathways (4, 5), is incompletely understood. There is growing evidence that suggests growth factors and cytokines play an important role in acquisition of hormone independence.

IL-6 is a glycoprotein consisting of 212 amino acids encoded by the IL-6 gene localized to chromosome 7p21-14 (6). IL-6 is a pleiotropic cytokine that plays a central role in host defense mechanisms by regulating immune responses, hematopoiesis, and the induction of acute phase reaction (6). The biological activities of IL-6 are mediated by the IL-6 receptor. The receptor for the IL-6 family of cytokines (IL-6, IL-11, ciliary neurotrophic factor, oncostatin M, and leukemia inhibitory factor) is composed of an IL-6-specific receptor subunit (α chain) and a signal transducer, gp130 [β chain (7)]. The binding of IL-6 to its receptor resulted in activation of intracellular signaling including Janus kinase-Stat and MAPK pathways (7, 8).

The expression and function of IL-6 in prostate cancer have been the subject of multiple recent studies. The expression of IL-6 and its receptor has been consistently demonstrated not only in human prostate cancer cell lines but more importantly in human prostate carcinoma and benign prostate hyperplasia obtained directly from patients (9-11). The levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer (12, 13). Furthermore, IL-6

<sup>4</sup> The abbreviations used are: IL, interleukin; AR, androgen receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PSA, prostate-specific antigen; Stat, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; ARE, androgen-responsive element; FBS, fetal bovine serum; CS, charcoal-stripped; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift analysis; PI3K, phosphatidylinositol 3'-kinase; ERK, extracellular signal-regulated kinase.

has been demonstrated as a candidate mediator of human prostate cancer morbidity (14). IL-6 has been suggested to have both growth-promoting and -inhibiting activities in androgen-dependent LNCaP human prostate cancer cells *in vitro*. IL-6 can function as a paracrine growth factor for the human LNCaP androgen-sensitive prostate cancer cells and an autocrine growth factor for human DU145 and PC3 androgen-insensitive prostate cancer cells (15–18). IL-6 can also function as a paracrine growth inhibitor for LNCaP cells and an autocrine growth stimulator for the DU145 and PC3 cells (19). Recently, results from a number of groups demonstrated that IL-6 activates AR-mediated gene expression in LNCaP cells *in vitro* (17, 20–22), suggesting that IL-6 may play a critical role during the progression of prostate cancer.

Whereas numerous studies have suggested the role of IL-6 in the growth and androgen responsiveness of prostate cancer cells *in vitro*, there is no experimental evidence to demonstrate the role of IL-6 in the promotion of androgen-independent growth of prostate cancer cells *in vivo*. In this study, we tested whether IL-6 induces androgen-independent growth. We demonstrate that IL-6 induces androgen-independent growth of androgen-sensitive LNCaP human prostate cancer cells both *in vitro* and *in vivo*, which is accompanied by elevation of PSA levels. The androgen-independent phenotype induced by IL-6 in LNCaP cells is mediated in large part by activation of Stat3 signaling and potentially also by activation of the MAPK pathway.

## MATERIALS AND METHODS

**Cell Culture.** The LNCaP cells were maintained in RPMI 1640 containing penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% FBS at 37°C in 5% CO<sub>2</sub> incubator. The IL-6-overexpressing cells (LN-S15 and LN-S17) and neo control (transfected with vector alone) cells were cultured in the same medium plus 0.3 mg/ml G418. To investigate the androgen withdrawal effect, cells were cultured in medium containing 10% CS-FBS instead of regular 10% FBS.

**In Vitro Cell Proliferation.** LNCaP cells or IL-6-overexpressing cells (LN-S15 and LN-S17; 10<sup>4</sup> cells/well) were plated in 12-well plates in RPMI 1640 containing 10% FBS. After 2 or 3 days in regular culture medium with 10% FBS, cells were switched into a medium of phenol red-free RPMI 1640 containing either 10% FBS or 10% CS-FBS (Hyclone). For controls, antihuman IL-6 antibody (20 µg/ml; Sigma) was added into the tissue culture medium. Two days later, cells were determined by using the MTT assay (Sigma) according to the manufacturer's instructions.

**In Vivo Tumor Growth.** Four- to six-week-old athymic male nude mice (Harlan, Indianapolis, IN) were inoculated s.c. in the flank with 3 × 10<sup>6</sup> cells (LNCaP, neo, LN-S15, and LN-S17) resuspended in Matrigel (BD Biosciences, Bedford, MA) diluted 1:1 in complete culture medium. The volume of the growing tumors was estimated by measuring three tumor dimensions (length × width × depth) with a caliper (23).

**RT-PCR.** RT-PCR was performed as follows. Briefly, total RNA was isolated from cells using the Trizol method (Life Technologies, Inc., Rockville, MD). One µg of total RNA was used in the reverse transcription reaction, and thermal cycling

was programmed as follows: 1 min at 4°C; 2 min at 70°C; and 5 min at 4°C with oligodeoxythymidine acid. After chilling tubes on ice, buffer, deoxynucleotide triphosphates, RNase inhibitor, and mouse mammary tumor virus were added and incubated at 42°C for 1 h. The cDNAs thus obtained were amplified with 30 cycles (45 s at 95°C, 1 min at 58°C, and 1 min at 72°C) of PCR reaction in the presence of Taq polymerase (Promega, Madison, WI). PSA primer sequences used were 5'-GGCAGGTGCTTGTAGCCTCTC-3' (sense) and 5'-CAC-CCGAGCAGGTGCTTTGC-3' (antisense). The PCR products were then resolved in a 1.5% agarose gel, and bands were analyzed with Molecular Imager FX System (Bio-Rad, Hercules, CA). GAPDH primers were used as control.

**Northern Blot.** Twenty µg of RNAs were electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 1.1-kb *Bam*HI fragment containing the PSA cDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol; ICN, Costa Mesa, CA) using Ready-To-Go DNA Labeling Beads (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Hybridization was carried out during 3 h at 65°C in Rapid-hyb buffer (Amersham Pharmacia Biotech). Membranes were washed for 15 min at 65°C in 2× SSC, 0.1% SDS (twice); 0.5× SSC, 0.1% SDS; and 0.1× SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio-Rad).

**Determination of PSA Secretion.** The serum was collected at the end of experiments. Fifty µl of serum were used to determine PSA secretion. Levels of PSA in the serum of tumor-bearing mice were determined by ELISA with the use of anti-PSA as primary antibody as described by the manufacturer's protocol (Beckman Coulter, Fullerton, CA).

**EMSA.** Whole cell extracts were prepared by using high-salt buffer [20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 420 mM NaCl, 20% glycerol, 1 µg/ml leupeptin, and 1 µg/ml aprotinin], followed by snap-freezing in ethanol/dry ice for 5 min and thawing on ice for 10 min. The freeze and thaw procedures were repeated again for a total of two times. The supernatant was then centrifuged and harvested. Protein concentrations were determined by Coomassie Blue plus protein assay kit (Pierce) according to the manufacturer's protocol. Stat3 DNA binding activity was determined by EMSA using Stat3 consensus oligonucleotide 5'-GATCCTTCTGGGAATTCCTAGATC as described previously (24). For determination of the AR DNA binding activity, whole cell extracts (20 µg) were incubated in a final volume of 20 µl [10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 µg/ml poly(deoxyinosinic-deoxycytidyl acid)] by EMSA with radiolabeled double-stranded AR consensus binding motif (Santa Cruz Biotechnologies, Santa Cruz, CA). The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25× Tris-borate EDTA at room temperature, and the results were autoradiographed. Quantitation of the amount of AR DNA binding activity in the "protein-DNA" bandshift was measured using the Molecular Imager FX System (Bio-Rad). For the supershift experiment, 20 µg of cell extracts were incubated with either Stat3 antibody or AR antibody

(Santa Cruz Biotechnologies) for 1 h at 4°C before incubation with the radiolabeled probe.

**Nuclear Lysate Preparation.** Nuclear protein extracts were prepared as described previously (17). Briefly, for nuclei preparation, cells were harvested, washed with PBS twice, resuspended in hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, and 0.1% NP40], and incubated on ice for 10 min. Nuclei were precipitated with 3,000 × g centrifugation at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100] and incubated on ice for 30 min. The nuclear lysates were precleared by 20,000 × g centrifugation at 4°C for 15 min. Protein concentration was determined by Coomassie Blue plus protein assay kit.

**Western Blot Analysis.** Forty µg of protein were resolved in 8–12% SDS-PAGE, depending on the molecular weight of the protein to be detected. After blocking overnight at 4°C in 5% milk in PBS-0.1% Tween 20, membranes were incubated overnight with antibodies against either Stat3, phosphorylated Stat3, p44/42ERK1/2, phosphorylated p44/42ERK1/2, Akt, phosphorylated Akt (Cell Signaling Technology) or AR (Santa Cruz Biotechnology). After secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

## RESULTS

### Effect of IL-6 on the Growth of LNCaP Cells *in Vitro*.

It was demonstrated previously that the androgen-sensitive LNCaP cells express IL-6 receptor but express no detectable IL-6 protein (15–19). IL-6 can enhance AR-mediated PSA expression in LNCaP cells (17, 18, 20–22), suggesting that IL-6 can enhance androgen responsiveness of LNCaP cells. To determine the effect of IL-6 on the growth of LNCaP cells in the presence and absence of androgen, we ectopically expressed IL-6 by introduction of a full-length IL-6 cDNA into IL-6-negative LNCaP cells as described previously (16). Several stable transfectants containing IL-6 cDNA in the sense orientation and vector-alone controls were selected in the presence of G418, subcloned, and tested for their expression of IL-6 by ELISAs. Two stable IL-6 transfectants (LN-S15 and LN-S17) expressing high levels of IL-6 (2465 and 2743 pg/ml/10<sup>6</sup> cells, respectively) were selected for additional studies. To test whether IL-6 promotes LNCaP androgen-independent cell growth *in vitro*, parental LNCaP, neo control, and IL-6-overexpressing clones were cultured in the presence and absence of androgen, and the cell growth was determined. As shown in Fig. 1A, the growth of androgen-sensitive LNCaP cells and neo control in culture was reduced by about 50% after 48 h in androgen-deprived CS serum (Hyclone; testosterone concentration is <10<sup>-11</sup> M, in which prostate epithelial cells do not respond to testosterone stimulation; Ref. 25) compared with that in normal serum (testosterone concentration is about 10<sup>-9</sup> M). Addition of dihydrotestosterone (10<sup>-9</sup> M) in the CS serum restored LNCaP cell growth to levels similar to that of the complete normal serum (data not shown). In the clones of LNCaP cells overexpressing IL-6 (LN-15 and LN-17), however, there was only a 5–10% decrease in growth under these andro-

gen-deprived conditions compared with growth in normal serum, suggesting that overexpression of IL-6 can enhance the growth of LNCaP cells in the androgen-deprived condition *in vitro*. Addition of anti-IL-6 antibody in IL-6 overexpression clones restored the growth inhibition to about 60% under androgen-deprived conditions compared with growth in normal serum.

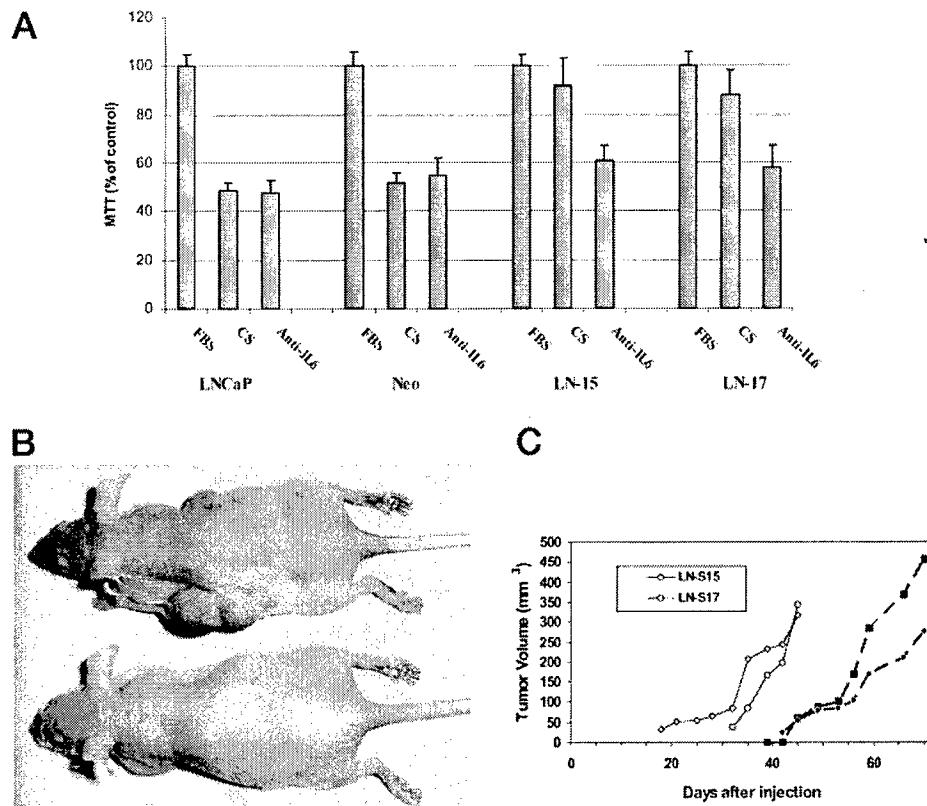
### IL-6 Induces Androgen-independent Growth *in Vivo*.

Having demonstrated that IL-6 enhances the growth of androgen-sensitive LNCaP cells in the absence of androgen *in vitro*, we further tested the effect of overexpression of IL-6 on the growth of androgen-sensitive LNCaP human prostate cancer cells *in vivo*. To test the effects of IL-6 on tumor formation and induction of androgen-independent growth of LNCaP cells *in vivo*, 8-week-old male nude mice were randomly divided into two groups; one group was left intact, and the other group received surgical castration, in which the residual levels of androgen are insufficient to maintain growth of androgen-sensitive LNCaP cells (1). Three days after castration, intact or castrated male nude mice were inoculated s.c. with parental LNCaP, vector control neo, or LNCaP cell clones overexpressing IL-6 with coinoculation of Matrigel. For the two independent IL-6-overexpressing clones, tumors became apparent at the site of injection within 30 days in the intact male mice and within 40 days in the castrated male mice (Fig. 1, B and C). Parental LNCaP cells and vector control neo clone did not grow any detectable tumor in both intact (within a 40-day observation period) and castrated (within a 70-day observation period) male nude mice. These results demonstrate that IL-6 promotes the growth of androgen-sensitive LNCaP cells in the absence of androgen *in vivo*.

### IL-6 Enhances Androgen-responsive Gene PSA Expression *in Vitro* and *in Vivo*.

Results from a number of groups demonstrated that IL-6 activates AR-mediated PSA gene expression in LNCaP cells *in vitro* (17, 20–22). To test whether overexpression of IL-6 enhances the expression of an endogenous, androgen-regulated PSA, the expression of PSA was compared between the parental and IL-6-overexpressing LNCaP cells in the presence and absence of androgen. As shown in the Fig. 2A, in the presence of androgen, PSA mRNA expression was elevated in the IL-6-overexpressing LNCaP cells compared with the parental and vector control LNCaP cells. When the cells were cultured in phenol red-free medium supplemented with the CS serum, in which the androgen was deprived, PSA mRNA expression was elevated in the IL-6-overexpressing clones compared with the parental and vector control LNCaP cells (Fig. 2B), suggesting that overexpression of IL-6 can enhance endogenous PSA expression in the presence and absence of androgen. These results are consistent with previous reports that IL-6 activates the PSA promoter/enhancer in the presence and absence of androgen (17, 20, 21). In addition, tumors generated from IL-6-overexpressing LNCaP cells also produced high levels of circulating PSA in the serum of both the intact male mice (average, 38 ng/ml per g of tumor) and the castrated male mice (average, 32 ng/ml per g of tumor).

To test whether IL-6 can influence the DNA binding activity of AR protein to the ARE, we performed EMSA using radiolabeled oligonucleotides of the ARE with nuclear extracts from LNCaP cell clones. LNCaP clones overexpressing IL-6



**Fig. 1.** Overexpression of IL-6 promotes LNCaP androgen-independent growth. *A*, effect of overexpression of IL-6 on LNCaP cell growth in the presence and absence of androgen *in vitro*. Cells were cultured in RPMI 1640 supplemented with 10% FBS. After 24 h, the cells were switched to either 10% FBS or 10% CS-FBS (CS). For controls, cells were cultured in RPMI 1640 supplemented with 10% CS-FBS plus 20  $\mu$ g/ml anti-IL-6 antibody. After incubation for another 72 h, MTT assays were performed. MTT values for the complete FBS were expressed as 100%, and MTT values for CS-FBS were expressed as a percentage relative to complete FBS. *B*, IL-6-overexpressing clone (LN-15) developed tumors *versus* parental LNCaP cells that did not grow any tumor in the castrated male nude mice. *C*, tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone or IL-6-expressing clones (LN-15 and LN-17) were injected into the intact (solid lines, open symbols) or castrated (dashed lines, filled symbols) male nude mice ( $n = 8$  for each condition). Parental LNCaP and neo control did not grow tumor.

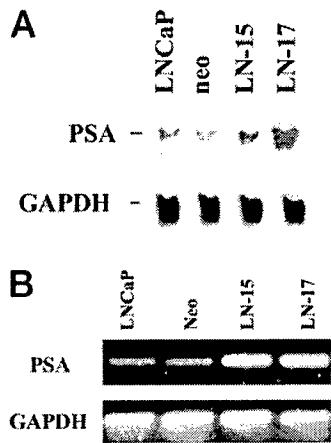
showed an increase in AR-ARE complex formation compared with the parental LNCaP cells in the presence of androgen as well as in the absence of androgen (Fig. 3A). The AR-ARE complex in the IL-6-overexpressing clones has little change in the absence of androgen compared with that in the presence of androgen (Fig. 3A). The specificity of this AR-ARE complex was demonstrated by supershift assay using antibody specifically against AR (Fig. 3B).

The AR typically translocates to the nucleus to exert its function on gene expression. To examine whether overexpression of IL-6 affects the expression and translocation of AR, Western blot analysis was performed using cell extracts from either whole cell extracts or nuclear extracts. As shown in Fig. 3C, overexpression of IL-6 in LNCaP cells significantly enhanced the expression of AR in the nuclear compartment without alteration of the total AR expression (whole cell extracts) both in the presence of androgen (FBS) and in the absence of androgen (CS-FBS).

**Overexpression of IL-6 Activates Its Downstream Signaling Pathways in LNCaP Cells.** The effects of IL-6 on prostate cancer cells are mediated by a variety of signal

transduction pathways including Janus kinase-Stat, MAPK, and PI3K-AKT pathways, resulting in proliferation, differentiation, and inhibition of apoptosis. To examine which pathways were altered by overexpression of IL-6 in LNCaP cells, cell lysis from parental and IL-6-overexpressing LNCaP cells were analyzed. We first examined the effect of overexpression of IL-6 on the expression and activation of Stat3, a major mediator of IL-6 signaling. As shown in Fig. 4A and 4B, overexpression of IL-6 significantly elevates the activity of Stat3 both in the presence of androgen (FBS) and in the absence of androgen (CS-FBS).

To determine whether the increased Stat3 activity is associated with increased Stat3 protein expression and elevated phosphorylation, Western blots of whole cell extracts from the parental LNCaP and IL-6-overexpressing clones were performed using antibodies specific against either phosphotyrosine Stat3 (Tyr-705) or total Stat3 protein. As shown in Fig. 4C, overexpression of IL-6 induces Stat3 phosphorylation in LNCaP cells without alteration of total Stat3 expression, which is consistent with the results shown that IL-6 induces Stat3 activation in LNCaP cells. Collectively, these results demonstrate that



**Fig. 2** Overexpression of IL-6 increases PSA mRNA expression in LNCaP cells. *A*, PSA mRNA expression in IL-6-overexpressing clones (LN-15 and LN-17), vector control (neo), and LNCaP cells cultured in normal FBS media and examined by Northern blot analysis using 20  $\mu$ g of total RNA. GAPDH is a control for equal loading. *B*, PSA mRNA expression in IL-6-overexpression clones (LN-15 and LN-17), vector control (neo), and LNCaP cells cultured in androgen-deprived CS-FBS media for 72 h. The PSA mRNA was examined by RT-PCR. GAPDH is a control for equal loading.

overexpression of IL-6 significantly elevates Stat3 signaling in androgen-dependent human LNCaP prostate cancer cells.

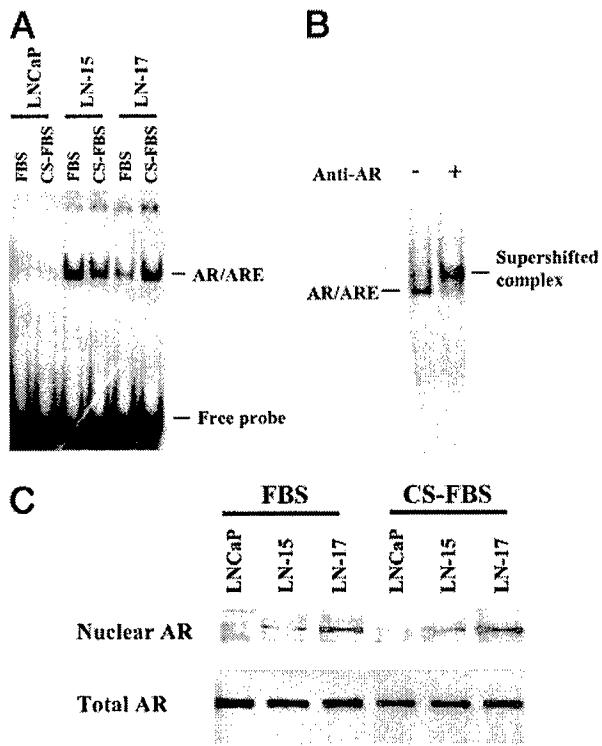
To investigate whether overexpression of IL-6 alters Akt or MAPK signaling pathways in LNCaP cells, we performed Western blot analysis on cell extracts from parental LNCaP and IL-6-overexpressing clones using antibodies that specifically recognize either phosphorylated Akt or phosphorylated MAPK (p44/42ERK1/2), respectively. As shown in Fig. 4*D*, overexpression of IL-6 in LNCaP cells enhances the levels of phosphorylated (active) p44/42 ERK1/2 expression without altering the expression of total p44/42 ERK1/2 in both the presence and absence of androgen, whereas overexpression of IL-6 in LNCaP cells has less effect on the expression of phosphorylated Akt or total Akt in both the presence and absence of androgen (Fig. 4*D*).

Collectively, these results indicate that IL-6-induced signaling in LNCaP cells is mediated primarily through Stat3 and MAPK signaling pathways.

## DISCUSSION

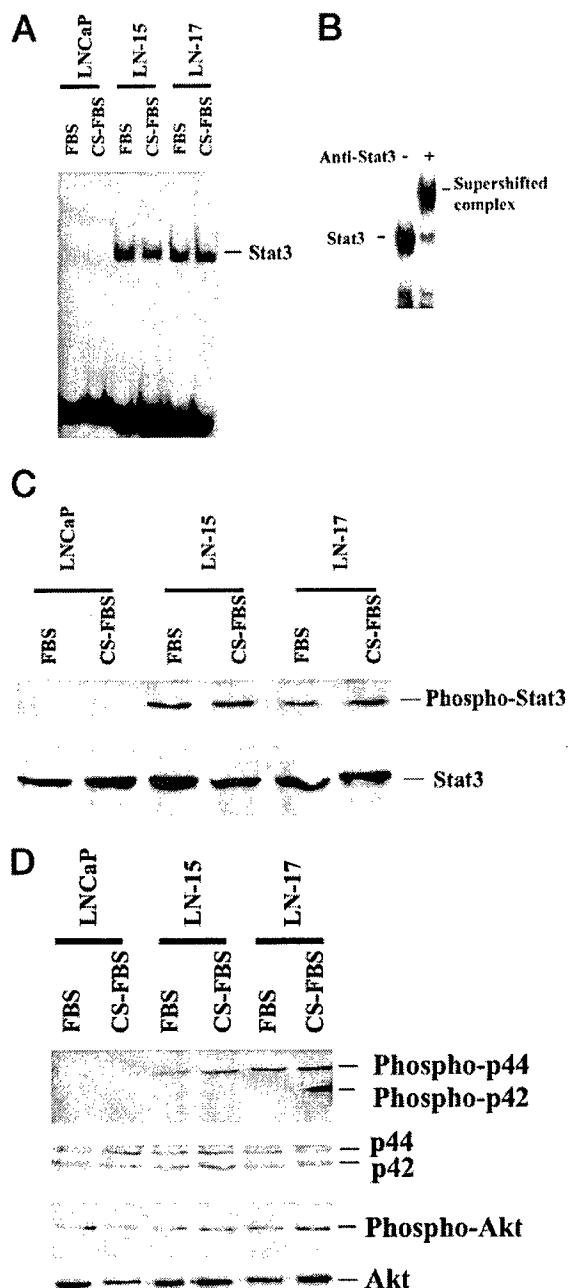
In the present study, we provide experimental evidence that IL-6 plays an important role in the induction of androgen-independent growth of human prostate cancer cells. We demonstrate that overexpression of IL-6 in androgen-sensitive human LNCaP prostate cancer cells results in the conversion of androgen-independent growth of LNCaP cells both *in vitro* and *in vivo*. Overexpression of IL-6 also enhances endogenous PSA expression in LNCaP cells, consistent with previous reports that IL-6 increases AR-mediated gene activation (17, 20–22). In addition, we demonstrate that IL-6 signaling is primarily mediated through activation of the Stat3 and MAPK signaling pathways in LNCaP cells.

The potential role of IL-6 in the development and progres-



**Fig. 3** Effect of overexpression of IL-6 on AR. *A*, IL-6 enhances the formation of AR-ARE complexes. EMSA was performed using radio-labeled ARE oligonucleotides with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. *B*, supershift assay of LN-17 cell extract using anti-AR antibody. Whole cell extracts were preincubated with antibodies specifically against AR as indicated. The positions of the AR-ARE and the supershifted complexes were indicated. *C*, overexpression of IL-6 enhances nuclear AR expression in LNCaP cells in the presence of androgen (FBS), but not in the absence of androgen (CS-FBS). Total cellular extracts and nuclear extracts were subjected to Western blot analysis (40  $\mu$ g/lane) using an antihuman AR antibody. The cells were cultured in RPMI 1640 with 10% FBS for 24 h and then switched to either 10% FBS or 10% CS-FBS, and culture continued for another 72 h. Cell lysates were extracted and used for the assays.

sion of prostate cancer cells has been suggested by numerous studies. Clinically, the levels of IL-6 in serum are significantly elevated in many men with advanced, hormone-refractory prostate cancer (12, 13). In addition, increased expression of IL-6 and IL-6 receptor has been demonstrated in prostate cancer tissues, and increased IL-6 receptor is correlated with increased proliferation of prostate cancer cells (9–11, 18). Experimentally, IL-6 has been suggested to have both growth-promoting and -inhibiting activities in androgen-dependent LNCaP human prostate cancer cells *in vitro* (16, 18, 19, 22). It has been demonstrated that IL-6 can act as a growth factor for both normal primary prostate epithelial cells and LNCaP prostate cancer cells *in vitro* (16–18). IL-6 stimulates prostate-specific protein expression in prostate carcinoma cells by activation of the AR and can be blocked by the antiandrogen bicalutamide (17, 20–22), consistent with our finding that overexpression of IL-6 enhances endogenous PSA expression in LNCaP cells. It



**Fig. 4** Overexpression of IL-6 activates the Stat3 and MAPK pathways. *A*, IL-6 induces Stat3 activation in LNCaP cells. EMSA was performed using radiolabeled Stat3 oligonucleotides with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. *B*, supershift assay of LN-17 cell extract using anti-Stat3 antibody. Whole cell extracts were preincubated with antibodies specifically against Stat3 as indicated. The positions of Stat3 and the supershifted complexes were indicated. *C*, overexpression of IL-6 enhances Stat3 phosphorylation in LNCaP cells. Western blots were performed using antibodies against either phospho-specific Stat3 (Tyr-705) or total Stat3 with whole cell extracts isolated from LNCaP and IL-6-overexpressing clones (LN-15 and LN-17) cultured in FBS and androgen-deprived CS-FBS media. *D*, the effect of overexpression of IL-6 on Akt and MAPK expression in LNCaP cells. Whole cell extracts from parental LNCaP cells and IL-6-overexpressing clones (LN-15 and LN-17) cultured in either normal FBS or CS-FBS conditions were subjected to Western blot analysis.

has also been indicated that IL-6 can mediate LNCaP cell growth arrest and induction of neuroendocrine differentiation (26, 27). Whereas all of the observed effects of IL-6 on the growth of prostate cancer cells were performed in tissue culture cells, mostly in androgen-dependent LNCaP human prostate cancer cells *in vitro*, the potential effects of IL-6 on LNCaP cells *in vivo* have not been reported. The present study is the first to provide such experimental evidence that IL-6 induces androgen-independent growth of androgen-sensitive human LNCaP prostate cancer cells both *in vitro* and *in vivo*. We have observed that overexpression of IL-6 in LNCaP cells significantly activates the Stat3 and MAPK signaling pathways. The observation of Stat3 activation by IL-6 is consistent with other reports that IL-6 stimulates prostate cancer cell growth through activation of the Stat3 signaling pathway (16, 18, 22, 27), and IL-6-induced activation of Stat3 in LNCaP cells increases AR-mediated gene activation in an androgen-independent but IL-6-dependent manner (20). IL-6 can activate erbB2 receptors, leading to activation of the MAPK pathway (22, 28). We also demonstrated that overexpression of IL-6 in LNCaP cells has less effect on the activation of Akt phosphorylation, which is different to the report that IL-6 can lead to activation of PI3K-Akt resulting in prevention of programmed cell death in human prostate cancer cell lines (18, 25, 29). The differential effects of IL-6 on the various signaling pathways (Stat3, MAPK, and PI3K-Akt) in LNCaP cells resulting in cell proliferation, differentiation, and survival are intriguing and are currently under intensive investigation.

PSA is a marker for prostate cancer, and the rise of the levels of PSA in the serum is an important indicator of prostate cancer progression. Several reports have indicated that IL-6 enhances PSA expression in LNCaP cells *in vitro* (17, 20–22), possibly through activation of Stat3 signaling (20). This is consistent with our finding that overexpression of IL-6 in LNCaP cells enhances endogenous PSA expression. In addition, we further demonstrated that overexpression of IL-6 induces PSA secretion to the serum in the castrated male nude mice, indicating that PSA levels induced by IL-6 are accompanied by LNCaP tumor growth in castrated male nude mice, similar to the clinical observation that rising PSA levels are a potential indicator of hormone-refractory prostate cancer. We have also demonstrated that overexpression of IL-6 enhances AR-ARE DNA binding activity and enhances AR nuclear translocation in LNCaP cells, which is consistent with the report that IL-6 increases AR expression in LNCaP cells (17).

One of the limitations of this study may be that IL-6 affects only LNCaP cells. LNCaP is an androgen-sensitive human prostate cancer cell line expressing a functional but mutant AR, which has been widely used for the study of prostate cancer. We are currently investigating the effect of IL-6 on androgen responsiveness in androgen-sensitive human prostate cancer cells

using antibody against phospho-specific p44/42 ERK1/2 and reprobed with total p44/42 ERK1/2 or antibody against phospho-specific Akt (p-Akt Ser473) and reprobed with total Akt. The cells were cultured in RPMI 1640 with 10% FBS for 24 h and switched to either 10% FBS or 10% CS-FBS, and culture continued for another 72 h. Cell lysis were extracted and used for the assays.

expressing a wild-type AR. Nevertheless, this study provides the first experimental evidence that IL-6 induces the transition of prostate cancer from an androgen-dependent to an androgen-independent phenotype, which corresponds to the induction of PSA expression through activation of AR. The androgen-independent phenotype induced by IL-6 in LNCaP cells is accompanied by significant activation of the Stat3 and MAPK signal pathways.

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